

Integrating Mechanical, Cell Geometric and Biochemical Inputs for Self-Organized Morphogenesis in Plant Regeneration

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मेबल मरिया माथ्यु / **Mabel Maria Mathew**

पंजीकरण सं. / Registration No.: **20193711**

शोध प्रबंध पर्यवेक्षक / Thesis Supervisor

डॉ. कालिका प्रसाद / **Dr. Kalika Prasad**



भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान पुणे

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"It is our choices that show what we truly are, far more than our abilities."

— J.K. Rowling, Harry Potter and the Chamber of Secrets

***Dedicated to
Amma, Papa, Kalika and God***

CERTIFICATE

Certified that the work incorporated in the thesis entitled “**Integrating Mechanical, Cell Geometric and Biochemical Inputs for Self-Organized Morphogenesis in Plant Regeneration**” submitted by **Mabel Maria Mathew** was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

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Date: 17-01-2025

Prof. Kalika Prasad

(Thesis supervisor)

DECLARATION

Name of Student: Mabel Maria Mathew
Reg. No.: 20193711
Thesis Supervisor: Dr. Kalika Prasad
Department: Biology
of joining program: 1st August 2022
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I declare that this written submission represents my idea in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I declare that I have acknowledged collaborative work and discussions wherever such work has been included. I also declare that I have adhered to all the principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

The work reported in this thesis is the original work done by me under the guidance of Prof. Kalika Prasad.

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Signature

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Mabel Maria Mathew



SYNOPSIS

Chapter 1

Chapter 1 introduces the complex process of regeneration using the model plant *Arabidopsis thaliana*, focusing on two broad categories of plant regeneration: tissue culture-induced regeneration and mechanical injury-induced regeneration. The former relies heavily on artificial culture conditions and can produce entire shoot and/or root systems from various plant parts, depending on the conditions provided. In contrast, mechanical injury-induced regeneration occurs without external hormones and involves responses such as vascular reconnection and root tip regeneration. Nevertheless, both categories of regeneration involve cellular reprogramming. In this chapter, I provide a comprehensive summary of the existing knowledge in the field. It is apparent that most literature on plant regeneration focuses on biochemical inputs such as genetic determinants, hormones, and molecular players. However, mechanical and geometrical inputs remain largely unexplored. The subsequent chapters offer an in-depth understanding of these mechanical and geometrical factors and how they interact with biochemical inputs to drive cellular reprogramming during plant regeneration.

Chapter 2

Chapter 2 details the cell biological, molecular, and genetic approaches used to investigate the mechanical, geometric, and biochemical inputs involved in cellular reprogramming during plant regeneration. These approaches include whole transcriptome

analyses, misexpression studies, and mutant analyses. Techniques such as confocal-based time-lapse imaging, laser-targeted cell ablation, Fluorescence Lifetime Imaging Microscopy (FLIM), and drug treatments were employed to study cell and tissue behavior. Additionally, various quantification methods and MorphoGraphX-based cell segmentation analyses were used to support the main conclusions.

Chapter 3

It is well-established that exposing plants to high levels of the hormone auxin induces the formation of a pluripotent, unorganized mass of cells known as callus. This callus can develop into either shoot or root systems depending on the external inductive cues provided. In the presence of auxin, the callus forms roots, whereas cytokinin, another plant hormone, promotes shoot formation. Interestingly, when cytokinin is externally applied, shoots arise from the callus in a stochastic manner, via a process known as *de novo* shoot regeneration. Previous studies in the lab revealed that these shoots originate from precursor cells called shoot progenitors, which emerge randomly within the unorganized callus. These progenitors expressed the polar auxin transporter PINFORMED 1 (PIN1) on their plasma membrane. This raised two key questions that intrigued me: (1) how specific callus cells were selected to become progenitors, and (2) what governed the progression of these progenitors into shoot meristems capable of forming complete shoot systems. By tracking the fate of hundreds of progenitors, my colleagues and I identified two distinct subpopulations: productive progenitors, which progressed into shoot meristems, and pseudo-progenitors, which failed and aborted midway. However, what distinguished productive progenitors from pseudo-progenitors remained an enigma. Eventually, we discovered that the critical factor was the

spatial localization of PIN1. Productive progenitors showed abundant and asymmetric PIN1 distribution along their plasma membranes, whereas pseudo-progenitors lacked this pattern. However, PIN1 was not the only protein that distinguished productive progenitors from pseudo-progenitors. I also discovered that intrinsic cell polarity proteins SOSEKI 1 (SOK1) and SOK2 exhibited distinct localization patterns between productive and pseudo-progenitors. Moreover, higher-order *sok1/2/3/4* mutants displayed severe defects in *de novo* shoot regeneration, despite showing normal embryonic and post-embryonic development. This finding suggested that *de novo* shoot formation follows a distinct pathway from embryonic shoot development. To further distinguish productive progenitors from pseudo-progenitors, I examined their mitotic activity. This analysis revealed that productive progenitors maintained higher and sustained division rates, whereas pseudo-progenitors ceased dividing and failed to progress into shoot meristems. While investigating mitotic activity, I discovered that progenitors predominantly emerged from regions of the callus with high cell division rates, known as morphogenic fields. This finding indicated that only cells within these mitotically active morphogenic fields were selected to become progenitors.

Lastly, considering the crucial role of stem cell regulators in development and regeneration, I investigated whether the abundance of the shoot stem cell regulator WUSCHEL (WUS) could distinguish productive progenitors from pseudo-progenitors. Surprisingly, WUS was equally expressed in both populations. This revealed that the mere abundance of stem cell regulators like WUS is insufficient to confer productive fate; instead, it is the spatial localization of polarity proteins that determines progenitor progression.

Chapter 4

Building on the findings from Chapter 3, where I established that the localization pattern of polarity proteins was crucial for *de novo* shoot regeneration, I wondered what promoted and maintained this localization pattern. In the lab, we had previously identified *ENDOTRANSGLYCOSYLASE/HYDROLASE 9 (XTH9)*, a gene encoding a cell wall loosening enzyme, as a key molecular player in promoting *de novo* shoot regeneration. However, I was puzzled by how a cell wall loosening enzyme could maintain the localization pattern of polarity proteins and promote shoot regeneration. My investigations revealed that XTH9 had a fascinating expression pattern: it was expressed exclusively in a shell of non-progenitor cells encapsulating the PIN1-marked progenitors. This spatial specificity of XTH9 was crucial, as its presence in the progenitor cells disrupted the PIN1 localization, impairing progenitor progression into shoot meristems. This led me to ask: What would happen if XTH9 activity was removed? Tracking the cells in the *xth9* mutant callus, I found that the non-progenitor cells surrounding the progenitors did not expand as they did in the wild type. This reduced expansion of non-progenitor cells was accompanied by the delocalization and diminishing of PIN1 from the progenitor plasma membranes, ultimately resulting in progenitor abortion. Not surprisingly, progenitors in the *xth9* mutant failed to progress to shoot meristems, and shoot regeneration was dramatically impaired. These findings emphasized the critical role of XTH9 expression in non-progenitor cells for maintaining PIN1 localization in progenitors and ensuring their successful progression into shoots.

Chapter 5

In Chapter 4, I discovered that XTH9 was exclusively expressed in the non-progenitor cells surrounding the progenitors. Given that spatial regulation of genes is known to drive localized differences in cell behavior (Gilmour *et al.*, 2017), I decided to investigate the behavior of progenitor cells in relation to their surrounding non-progenitor cells. Cell lineage tracing over a 24-hour period revealed that the progenitor cells were actively proliferating, while the surrounding non-progenitor cells rarely divided. Combining cell lineage tracing with volumetric analysis, I found that progenitor cells grew faster and more than the non-progenitor cells. This analysis also showed that progenitor cells primarily grew through cell division, while the non-progenitor cells expanded mainly through cell enlargement. I wondered what impact the differential growth rates between adjacent cells might have on their behavior. Within the tissue, if adjacent cells grow at different rates, there would be pull and push forces that could impact cell geometry (LeGoff & Lecuit, 2015; Dorothee *et al.*, 2022). Reasoning that the progenitors grew faster, I monitored and quantified the cell geometries of both the progenitor and non-progenitor cells. The results revealed that the progenitor cells were mostly isotropic, while the surrounding non-progenitor cells were elongated and expanded tangentially. This suggested that the progenitor and non-progenitor cells were under different mechanical stress fields, a speculation I soon confirmed by examining the microtubule (MT) orientation. The progenitor cells exhibited isotropic MT orientation, while the non-progenitor cells showed an anisotropic, circumferential pattern. This difference indicated mechanical heterogeneity between the two cell types.

Curious about the significance of this mechanical heterogeneity, I investigated the consequence of interfering with it. I later discovered that interfering with this mechanical difference by misexpressing XTH9 impaired the PIN1 localization pattern in progenitors. Similarly, physically disrupting the mechanical heterogeneity also led to impaired PIN1 localization and progenitor progression. This was evident when I found that removing XTH9-expressing cells resulted in the loss of PIN1 localization in the progenitor cells. Interestingly, this was not a one-way interaction, as removing progenitor cells led to the disappearance of XTH9 from the surrounding cells. This suggested a feedback loop between the progenitor and non-progenitor cells. These findings prompted me to propose a self-perpetuating model to explain self-organized morphogenesis. In this "rubber-shell" model, I hypothesized that non-progenitor cells act like a constraining rubber shell around the progenitors, maintaining their compact packing and PIN1 localization as the progenitors grow. The growth of the progenitors generates enough radial pressure to stretch the surrounding non-progenitor cells, which in turn accommodates further progenitor growth. This creates a cycle: progenitor growth expands the shell, and the shell expansion facilitates more growth in the progenitors, while maintaining PIN1 localization and the progenitor's structural integrity. This feedback loop likely drives progenitor progression and eventually leads to shoot meristem formation.

To test this model, I performed an ablation experiment where I removed a progenitor cell. I found that adjacent progenitor cells expanded and quickly filled the gap. When I removed non-progenitor cells to create a gap in the shell of XTH9-expressing cells, the adjacent PIN1-marked progenitor cells bulged outward and appeared to fall apart. These

observations confirmed that the shell of non-progenitor cells constrains the progenitor while also stretching to accommodate progenitor growth. This model helped explain why, in the *xth9* mutant, where the shell of non-progenitor cells fails to expand, progenitor growth halts and the progenitors eventually abort. In this chapter, my investigations revealed that the mechanical conflict between neighboring progenitor and non-progenitor cells is essential for maintaining the localization pattern of polarity proteins in progenitors and promoting shoot regeneration.

Chapter 6

In the previous chapter, I established that mechanical heterogeneity played a key role in *de novo* shoot regeneration. Building on this, I decided to examine the mechanical properties across the callus tissue. To do this, I created a comprehensive microviscosity map of the callus, encompassing both progenitor and non-progenitor cells, using a plant-specific rigidochromic dye called N⁺-BODIPY, which targets the plasma membrane. My investigations revealed that the plasma membranes of progenitor cells were more viscous, indicating compression compared to their surrounding non-progenitor cells. I also noticed that this compression varied along the z-axis, with deeper layers of progenitor cells exhibiting increased microviscosity. Furthermore, the microviscosity levels differed between the sides of each cell, highlighting mechanical heterogeneity at both cellular and subcellular levels.

This observation made me wonder if progenitors in the *xth9* mutant would have even higher microviscosity. As I anticipated, in the *xth9* mutant, where the expansion of the non-

progenitor cell shell was impaired, the microviscosity of the plasma membrane in the progenitor cells was significantly elevated. This increase in microviscosity was not limited to changes in cell wall properties; it also responded to alterations in turgor pressure. I discovered that after applying a hyperosmotic shock, the microviscosity of the plasma membrane increased, and the progenitor cells lost the expression of intrinsic cell polarity proteins, such as SOK. Eventually, these progenitors aborted. To further substantiate the role of mechanical properties in *de novo* shoot regeneration, I employed a genetic approach focused on mutants defective in cell wall properties and cytoskeletal components. As expected, shoot regeneration was impaired in these mutants, confirming the critical role of mechanical properties in the process. In this chapter, I demonstrated that the callus is mechanically heterogeneous at both cellular and subcellular levels, and that shoot regeneration from callus is hypersensitive to modulation of the mechanical properties of the callus.

Chapter 7

Soon, I realized that the importance of mechanics and cell geometry extended beyond *de novo* shoot regeneration—it was also crucial for the restoration of an injured root tip. When the root tip of *Arabidopsis* is excised, it loses its characteristic tapering shape, leaving behind a flat and straight end. The complete restoration of the root tip involves not only the regeneration of lost cells and tissues, but also the restoration of its tapering shape. In this chapter, I focused on investigating the restoration process of the injured root tip and uncovered a two-step mechanism that facilitated the restoration of its tapering shape. During my investigation, I came across a transient population of spatially specific cells with

rhomboid geometry located above the cut end. These cells were primarily situated in the cortex, between the outer epidermis and inner stele. What intrigued me most was that these cells were initially cuboid at the time of root tip excision, but they deformed into rhomboid shapes afterward. I hypothesized that this deformation occurred because the stele and/or endodermal cells grew faster than the surrounding tissue, pulling on the inner side of the cortical cells while the outer epidermis remained mostly stationary, growing more slowly. This likely created a mechanical strain that reshaped the cuboid cells into rhomboid shapes. This hypothesis was confirmed through the analysis of growth rates in different cell files of a regenerating root, which showed that the stele and endodermis indeed grew faster, while the epidermis grew significantly slower. This was further supported by 2D finite element simulations, which successfully recapitulated the formation of rhomboid shapes in the cortex when stele cells were stimulated to grow faster and epidermal cells grew slower.

The formation of rhomboid cell geometry marked the first step of the restoration process. The second step involved the functional role of these rhomboid cells in restoring the tapering shape of the injured root. I observed that these rhomboid cells underwent a unique diagonal division, producing two triangular-prism-shaped daughter cells. One of these cells oriented outward, while the other was directed inward at an inclined angle. Further investigations revealed that this peculiar division was tightly linked to the cell geometry, and its functional relevance lay in restoring the tapering shape of the root. I found that these diagonally dividing cells played a crucial role in deflecting the vertically growing neighboring cell files, redirecting them into inclined paths. This deflection drove the progressive tapering in the regenerating root. To test the necessity of these rhomboid cells, I

selectively removed a subset of these cells through ablation. This resulted in a significant delay in tapering, with the flat morphology at the cut end persisting until new rhomboid cells were formed and resumed the tapering process. Overall, my findings in this chapter highlighted a two-step mechanism for root tip restoration following injury where, (1) unequal growth between cell files induced the formation of rhomboid cortical cells, and (2) these rhomboid cells underwent diagonal division to mechanically guide surrounding cells, initiating tapering during root tip restoration. This previously unknown shape-forming mechanism emphasized how local geometric inputs drive tapering, offering fresh insights into organ shape restoration in plant regeneration.

Chapter 8

Building on the findings from Chapter 7, I sought to understand what facilitates the transformation of cuboid cells at the cut end into rhomboid geometry. While several factors could be at play, I focused on the biochemical aspects of this process. Specifically, I investigated how changes driven by PLETHORA 2 (PLT2) contributed to cell deformation and root tip restoration following injury. I chose PLT2 for my investigations because it is a plant-specific transcription factor (TF) expressed in a gradient from the distal to the proximal axis of the root, exhibiting dose-dependent activity (Galinha *et al.*, 2007; Santuari *et al.*, 2016). A previous study from the lab had shown that PLT2 levels set the boundary for root tip regeneration potential. Regeneration occurred when the root tip was excised at a position with high PLT2 levels (i.e., low cut), but not when excised at a position with low PLT2 levels (i.e., high cut). Moreover, overexpression of PLT2 for a brief period before excision extended the gradient proximally and promoted regeneration even from high cuts. However,

sustained overexpression of PLT2 abolished the regeneration potential, emphasizing its dosage-specific activity (Durgaprasad *et al.*, 2019).

In my own investigations, I found that high levels of PLT2 prior to injury were crucial for the formation of the transient, spatially specific rhomboid-shaped cells above the cut end (described in Chapter 7). In the *plt1,plt2* double mutant, where PLT2 was absent, these rhomboid cells failed to form, and root tip regeneration was completely lost. While brief overexpression of PLT2 did promote root tip regeneration from high cuts, I found that overexpressing PLT2 prior to the injury was essential. Overexpression immediately after the injury did not trigger regeneration, highlighting the need for elevated PLT2 levels before injury. This led me to wonder whether the levels of PLT2 influenced the biochemical environment of the cells. I investigated this and found that PLT2 modulated the expression of WOX5, a key marker of the quiescent center (QC) in the stem cell niche. Short-term overexpression of PLT2 prior to high cut allowed WOX5 to re-establish in the regenerating tissues, supporting restoration. However, prolonged PLT2 overexpression resulted in the loss of WOX5 and rhomboid cell generation, and abolished regeneration. This suggested that both the timing and dosage of PLT2 were critical for shaping the biochemical environment of the cells. To explore how PLT2 affects the broader genetic landscape, I conducted a comparative whole transcriptome analysis between injured roots that had undergone a brief period of PLT2 overexpression and those with sustained overexpression prior to injury. The analysis revealed genes involved in hormone signaling and cell wall organization. To perturb hormone signaling, I treated the injured roots with external cytokinin (a known shoot hormone). This treatment reduced the number of rhomboid cells and impaired root tip

restoration, indicating an interaction between biochemical and mechanical inputs during regeneration. In summary, this chapter emphasizes the relevance of pre-injury PLT2 levels for the generation of rhomboid cells and the restoration of the root tip.

Chapter 9

In this chapter, I discussed the convergence of three key inputs: mechanics, cell geometry, and biochemistry in plant regeneration. I explored how these three factors operate during the self-organized morphogenesis of *de novo* shoot meristem formation and the restoration of the root tip after injury. Looking ahead, these discoveries paved the way for understanding how these inputs contribute to self-organization, offering valuable insights into morphogenesis, development, and regeneration across biological systems.

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ABBREVIATIONS

0 H- 0 Hour	GFP- Green Fluorescent Protein
4 H- 4 Hour	GR- Glucocorticoid Receptor
6 H- 4 Hour	mm- Millimeter
8 H- 4 Hour	MS- Murashige and Skoog Medium
12 H- 4 Hour	ns- Nanosecond
24 H- 4 Hour	PI- Propidium Iodide
48 H- 4 Hour	PID- PINOID
AM- Axillary Meristem	PIN- PINFORMED
AP2- APETELLA 2	PLT- PLTETHORA
<i>At- Arabidopsis thaliana</i>	QC- Quiescent Centre
BDP- BODIPY	RAM- Root Apical Meristem
CFP- Cyan Fluorescent Protein	RIGED- Resection Induced Geometrically Deformed
CIM- Callus Induction Medium	SAM- Shoot Apical Meristem
CUC- CUP SHAPED COTYLEDON	SCN- Stem Cell Niche
D- Day	SEM- Standard Error of Mean
DDGs- Differentially Downregulated Genes	SIM- Shoot Induction Medium
DEGs- Differentially Expressed Genes	SOK- SOSEKI
DEX- Dexamethasone	TZ- Transzeatin
Dpc- Days post cut	WT- Wildtype

Dpg- Days post germination

WUS- WUSCHEL

DUGs- Differentially Upregulated Genes

XTH- XYLOGLUCAN

ENDOTRANSGLUCOSYLASE/HYDROLASE

YFP- Yellow Fluorescence Protein

μm- Micrometer

μm- Micromolar

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ABSTRACT

Morphogenesis in multicellular organisms relies on mechanical forces, cell geometry, and biochemical cues such as hormones, and these elements must act in concert. Using *Arabidopsis thaliana* as a model, I investigated these aspects of morphogenesis, and uncovered how they contribute to the *de novo* formation of a shoot meristem and the regeneration of a root tip. Through my investigations of the fundamental processes governing tissue culture-mediated shoot regeneration in *Arabidopsis*, I discovered the critical role of mechanical forces in the making of a shoot meristem *de novo*. The *de novo* shoot meristem is made from a select few cells, named shoot progenitors, from a large pool of undifferentiated mass of cells, called callus. I found that a cell-wall-loosening enzyme, XTH9, was exclusively expressed in a shell of cells surrounding the progenitor. This expression created a mechanical heterogeneity, with progenitor cells experiencing compression and their neighbours experiencing tension. This conflict in mechanical forces activated intrinsic cell polarity proteins and established the biochemical environment necessary for shoot meristem formation. Based on these, I propose a "stretch-compress" model to illustrate how these mechano-biochemical effects instruct formation of proper progenitors, and their progression into complete shoot systems. I further investigated the acquisition of a specific cell geometry and its role in wound repair using root tip regeneration as an experimental system. The plant roots are tapering to support soil penetration, and this tapering portion of the root tip is surgically removed during root tip resection. I investigated how the damaged plant roots regain their tapered shape, and suggest a two-step process driving this repair. I discovered that the first step featured the generation of a specific rhomboid-shaped cell geometry, arising from uneven growth between neighbouring cells. The second step uses this specific cell geometry as a template to establish arrangements, such as a unique diagonal division plane and redirection of the vertical longitudinal cell files into inclined trajectories. This redirection narrows the root's diameter, re-establishing the tapering shape of the root. I found that a gradient-expressed transcription factor (TF) conditioned the cells for their deformation into rhomboid shapes. This previously unknown shape-forming mechanism highlights how local cell geometries trigger tapering, offering

fresh insights into organ shape regeneration in plants. In summary, my study highlights the fundamental role of cell geometry and mechanochemical feedback as key regulators of tissue morphogenesis, offering new insights into the forces driving the developmental processes in plant regeneration.

Chapter I: Introduction and review of literature

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I. 1. INTRODUCTION

"If I have seen further, it is by standing on the shoulders of giants."

— Sir Isaac Newton

All living organisms share an inherent ability irrespective of their evolutionary origin, i.e. the ability to grow. In animals, growth is broadly characterised as the enlargement of a body plan that is established during embryogenesis. However, plants make a very limited set of structures during embryogenesis, and most of the plant organs that we see (e.g. the shoot and root) are made and grown post-embryonically. This growth is driven by groups of highly dividing cells, termed meristematic cells, located at the oppositely placed poles of the plant body axis: the shoot apical meristem (SAM) and the root apical meristem (RAM). The two meristems harbour a region called the stem cell niche (SCN) in which stem cells are confined. These stem cells act as self-perpetuating reservoirs of cells for making various organs during post-embryonic development. Plant growth and survival are compromised when these post-embryonic structures encounter frequent injuries, be it biotic or abiotic. To surpass the damaging effects of these injuries, plants elicit prompt regenerative responses.

Unlike most animals, in which regenerative responses are restricted to specific cell lineages, plants exhibit responses that are quite ubiquitous. As such, plants are distinct by virtue of their ability to regenerate specific cell types, tissues and organs, or even an entire organism. In addition, plants have the remarkable ability to rejoin two different body parts from two individual plants of different origin via grafting to form a ‘chimera’ that displays

the phenotype of both plants. Most importantly, plants display unparalleled plasticity despite the lack of cell migration, which is often key to regeneration in animals. Thus, the extreme fantasies of regeneration paraded by many mythical and comic characters are brought to life by one single life form: the plant.

Regeneration has been studied in a variety of plant species, revealing varying degrees of regenerative capacities and modes of regeneration. Here, we provide an overview of how an array of regenerative responses are being studied using the model plant species *Arabidopsis thaliana*, and how rapidly growing genetic, cellular and genomic tools have begun to unravel the mechanisms underlying these regenerative responses. We also introduce how a combination of experimental and computational approaches can allow us to gain deeper insights into broader questions in the area of plant regeneration.

I. 2. Arabidopsis as a model plant

Arabidopsis thaliana (Figure. 1.1), by virtue of its small genome size, ease of cultivation, short life-span and prolific seed production, has long served as a model in plant molecular and genetic studies. In recent years, the use of *Arabidopsis* has been substantiated by extensive physical and genetic maps of its chromosomes, high-resolution expression maps in space and time, and spatio-temporal studies of various molecular interactions (e.g. protein-protein interactions and protein-DNA interactions) at cellular resolution in its shoot and root (Cui *et al.*, 2007; Brady *et al.*, 2007; Weigel & Mott, 2009; Busch *et al.*, 2012; Kulkarni *et al.*, 2018). In addition to a vast repository of mutant lines, full length cDNAs and miRNA collections, inducible knockdowns and knockouts of desired genes using RNAi and

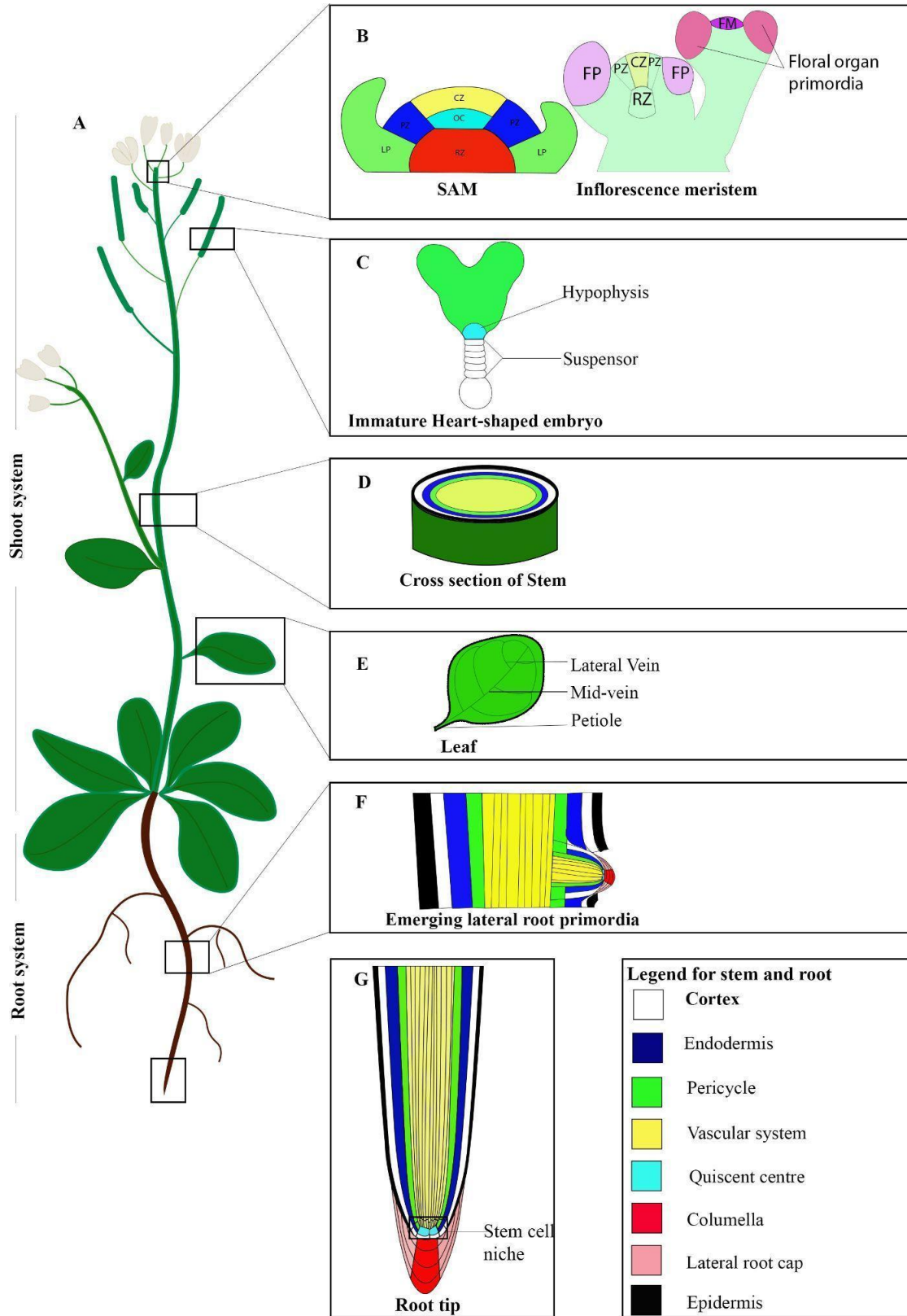


Figure. 1.1. Arabidopsis plant architecture. (A) Representative image of a whole plant. (B) Upon transition from the vegetative phase to the reproductive phase, the shoot apical meristem (SAM), which produces a leaf at its flank, becomes an inflorescence meristem and produces a flower at its flank. The organizing centre (OC) and the central zone (CZ) within the SAM constitute the stem cell niche (SCN). (C) Inset depicts an immature heart-shaped embryo within the silique (fruit). (D) Inset displays a cross-section of the inflorescence stem showing different tissues. (E) Inset shows the leaf with mid vein, lateral vein and petiole. (F) Inset depicts lateral root emergence. (G) Inset depicts the root tip showing different tissues. The quiescent centre and its surrounding cells form the SCN within the root apical meristem. FM, floral meristem; FP, floral primordia; LP, leaf primordia; PZ, peripheral zone; RZ, rib zone.

CRISPR-Cas9 have been created and made available (Mao *et al.*, 2019; Schwab *et al.*, 2006). A recently developed technology, termed Inducible Genome Editing (IGE), which knocks out target genes in specific cell types at any developmental age, has further advanced the strengths of *Arabidopsis* as a model plant (Wang *et al.*, 2020b).

The genome-wide binding of a variety of key regulators and their direct downstream targets has also been made available in *Arabidopsis* using DAP-seq, a high-throughput transcription factor (TF) binding site discovery method (O'Malley *et al.*, 2016). In addition, highly sensitive sensors that can measure fluctuations in hormone levels in *Arabidopsis* have been developed (Brunoud *et al.*, 2012; Kang *et al.*, 2012; Liao *et al.*, 2015). The readily available molecular, genetic and cell biological tools, together with computational models and the wealth of knowledge of embryonic shoot and root development, has made *Arabidopsis* the perfect choice for studying the mechanisms of cellular reprogramming during normal development as well as during regeneration (Mündermann *et al.*, 2005; Smith *et al.*, 2006; Grieneisen *et al.*, 2007; Cruz-Ramírez *et al.*, 2012; Sampathkumar *et al.*, 2014).

I. 3. Types of regenerative modes in Arabidopsis

Arabidopsis shows a repertoire of regenerative responses that can be as elaborate as regenerating an entire plant from a small tissue or regenerating a lost organ, or as simple as inducing a wound healing response following mechanical injury. These modes of regeneration fall into two broad categories, as highlighted below.

I.3.1. Tissue culture-induced regeneration

Plants can regenerate themselves from various explants of different developmental origins. An explant is a piece of tissue or organ that can be cultured *in vitro* to regenerate organs or an organism *de novo*. This property of explants to produce entire organisms can be exploited for large-scale seedling production. Here, an entire plant is regenerated through the sequential regeneration of shoots and roots. Formation of such organs from explants *in vitro* is called *de novo organogenesis*, and can further be classified into *de novo* shoot regeneration (i.e. shoot formation) and *de novo* root regeneration (i.e. root formation). *de novo organogenesis* can occur via two different pathways, namely direct and indirect regeneration. During indirect regeneration, the explants produce a shoot/root via an intermediary stage called a callus (Figure. 1.2). In direct regeneration, by contrast, the explants can bypass this callus stage to produce a shoot/root. For example, shoot regeneration can occur from lateral root primordia (LRP) (Figure. 1.3.A, 1.3.B).

In addition to shoots and roots, explants can regenerate embryos and thereby the entire plant system. Here, somatic cells from explants can be induced and cultured *in vitro* to generate somatic embryos via the process of somatic embryogenesis. Similar to *de novo*

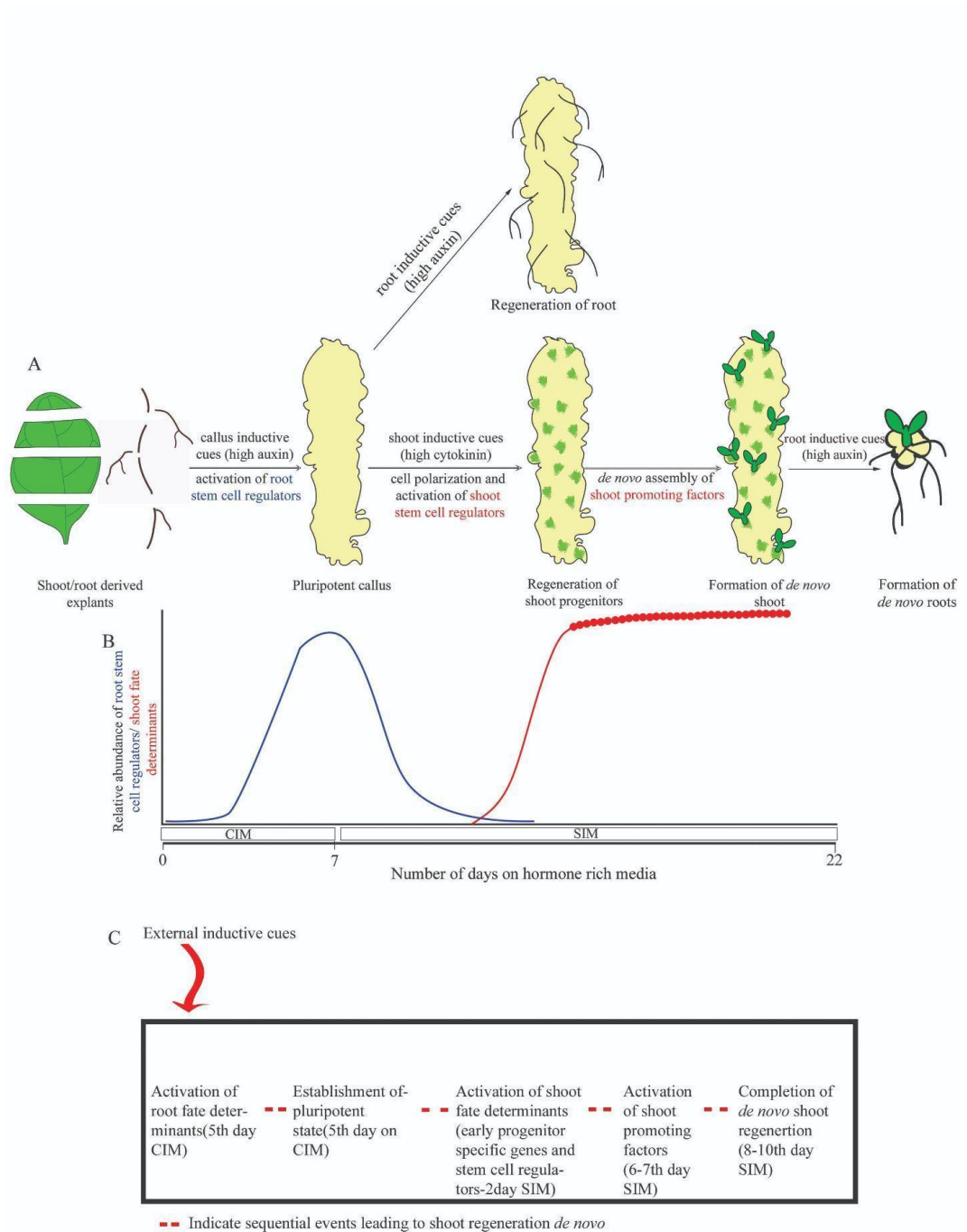


Figure. 1.2. Indirect tissue culture-induced regeneration. (A) Summary of the events that occur during tissue culture-mediated root (top) and shoot (bottom) regeneration. Explants of different developmental origins first form a pluripotent callus. They then undergo self-organization and, if cultured in the presence of shoot inductive cues, form shoot progenitor cells, ultimately culminating in the formation of a shoot (bottom). Note that not all shoot progenitors convert into shoots; if explants are cultured in root inductive cues, root formation

occurs (top). **(B)** Graph depicting the relative abundance of root stem cell regulators and shoot fate determinants (y-axis) against the days (x-axis) of explant culture on callus induction medium (CIM) or shoot induction medium (SIM). Root stem cell regulators (blue) are transiently upregulated throughout the callus while it is incubated on CIM, ensuring acquisition of pluripotency, and are downregulated following transfer of the callus onto SIM. In response to shoot inductive cues, shoot fate determinants (red) get activated. Their expression peaks by the time the callus undergoes shoot progenitor regeneration and self-assembly (denoted by broad red dots), leading to the formation of an entire shoot *de novo*. **(C)** Regulatory module highlighting the sequential events occurring during regeneration of a shoot system *de novo*.

organogenesis, somatic embryogenesis can also occur indirectly via a callus or directly from the explant (Figure. 1.3.C) (Pillon *et al.*, 1996; Mordhorst *et al.*, 1998).

I.3.2. Mechanical injury-induced regeneration

Plants are constantly subjected to mechanical injuries by biotic factors such as herbivores, insects and nematodes, as well as abiotic factors such as strong winds and heavy rains. Mechanical injury-induced regeneration can be exhibited by an organ in two scenarios: (1) when the damaged organ is still attached to the parent plant; (2) when the organ is detached from the parent plant.

The most common scenario involves the functional restoration of a damaged organ that is attached to the growing plant. Both aerial and underground organs are capable of combating these injuries. Injuries to aerial organs include vascular disruption inflicted by sap-feeding insects, breakage of stems by strong winds, or even surface abrasion due to friction. Friction-induced surface abrasion usually does not pose a major threat and is healed by local cell proliferation. However, injury to the plant vasculature impedes the transport of water and nutrients along the plant body axis, and prompt re-establishment of vascular continuity is therefore imperative. Injuries to the root are often caused by soil-dwelling nematodes. The

injuries range from damage of individual cells by the proboscis of insects to even the loss of an entire root tip. As roots mediate the absorption of water and nutrients, plants initiate immediate defense and regenerative responses to repair this damage.

In contrast to an attached organ, a detached organ exhibits entirely different regeneration responses. Instead of repair and functional restoration of the existing tissue, a detached organ has the ability to regenerate a new organ with an entirely different identity from its cut end. For example, *de novo* root regeneration can occur from the cut ends of detached *Arabidopsis* leaves. Interestingly, a detached organ from one plant can be attached to another plant via the process of grafting to generate a single fully functional plant with characteristics from both the combining plants.

I.4. Tissue culture-induced regeneration

I.4.1 *De novo* shoot regeneration via callus formation

An entire plant can be regenerated from explants of different developmental origin (Glazebrook & Weigel, 2002). These explants, when incubated with synthetic auxin, produce a pluripotent callus from xylem pole pericycle (XPP) cells that, during normal development, give rise to lateral roots (LRs) (Malamy & Benfey, 1997; Atta *et al.*, 2009; Sugimoto *et al.*, 2010). The molecular nature of this callus has been studied extensively to reveal the regulators controlling the formation of a callus and its acquisition of pluripotency.

During callus formation, one of the regulators of LR formation, ABERRANT LATERAL ROOT FORMATION 4 (ALF4), plays a crucial role (DiDonato *et al.*, 2004).

ALF4 modulates auxin signalling and is essential for LR initiation (Bagchi *et al.*, 2018); it is this involvement in the auxin response that possibly makes ALF4 crucial for callus formation. Interestingly, the callus predominantly expresses root-specific genes (Sugimoto *et al.*, 2010). Although callus formation necessitates activation of ALF4, pluripotency acquisition by the callus requires the recruitment of root stem cell regulators such as PLETHORA (PLT)1, PLT2, SCARECROW (SCR) and WUSCHEL RELATED HOMEODOMAIN 5 (WOX5) (Figure. 1.2.B) (Sugimoto *et al.*, 2010; Kareem *et al.*, 2015; Kim *et al.*, 2018). The onset of the expression of root stem cell regulators is under the control of several genetic and epigenetic regulators (Kareem *et al.*, 2015; Kim *et al.*, 2018).

Once a pluripotent callus has formed, its further fate in regeneration is decided by two major plant hormones: auxin and cytokinin. When supplemented with a higher auxin: cytokinin ratio the callus initiates root regeneration, whereas when supplemented with higher cytokinin: auxin it initiates shoot regeneration (Figure. 1.2.A) (Skoog & Miller, 1957). Shoot regeneration is fine-tuned by two groups of regulators of cytokinin signalling, namely Type A Arabidopsis response regulators (ARRs), which are negative regulators, and Type B ARRs, which are positive regulators (for a review see (To & Kieber, 2008)). Shoot regeneration necessitates the activation of Type B ARRs and the removal of epigenetic repression marks from

shoot stem cell regulators (Zhang *et al.*, 2017; Meng *et al.*, 2017). The resulting cytokinin signalling response activates shoot stem cell regulators and several shoot-promoting factors, thereby facilitating *de novo* shoot regeneration (Figure. 1.2.B) (Atta *et al.*, 2009; Kareem *et al.*, 2015; Iwase *et al.*, 2017). In the process, shoot initials called progenitors are formed

before the formation of a functional shoot meristem. Although all cells of the callus are theoretically pluripotent, shoot progenitors do not initiate from all cells but rather arise stochastically from a few cells of the callus. They can be identified using fluorescent-labelled markers that mark the polar auxin transporter PINFORMED (PIN1) and the shoot stem cell regulator WUSCHEL (WUS) (Gordon *et al.*, 2007; Zhang *et al.*, 2017). Further, not all the shoot progenitors can make it to completion of shoot regeneration, indicating that the callus is a highly heterogeneous mass of cells (Gordon *et al.*, 2007; Motte *et al.*, 2014; Radhakrishnan *et al.*, 2018; Lardon *et al.*, 2020).

In response to shoot inductive cues, root-specific genes that are instrumental in generating a pluripotent landscape get downregulated, with a concomitant increase in shoot-promoting factors, thereby facilitating the onset of shoot regeneration. The journey from acquisition of pluripotency to completion of shoot regeneration involves an interesting two-step molecular mechanism comprising three redundantly acting PLT genes, namely, PLT3, PLT5 and PLT7. These PLT genes control lateral organ positioning during normal root and shoot development (Prasad *et al.*, 2011; Hofhuis *et al.*, 2013). In the first step, the PLT genes activate root stem cell regulators, making the callus competent to regenerate shoot progenitors. In the second step, which is dependent on the first step, shoot-promoting factors such as CUP-SHAPED COTYLEDON 2 (CUC2) are activated by the PLT genes to accomplish regeneration of the complete shoot system (Kareem *et al.*, 2015) (Figure. 1.2.C). Acquisition of competency for shoot regeneration is thus uncoupled from the completion of shoot regeneration from a callus. This two-step mechanism operates in all explants irrespective of their developmental origin.

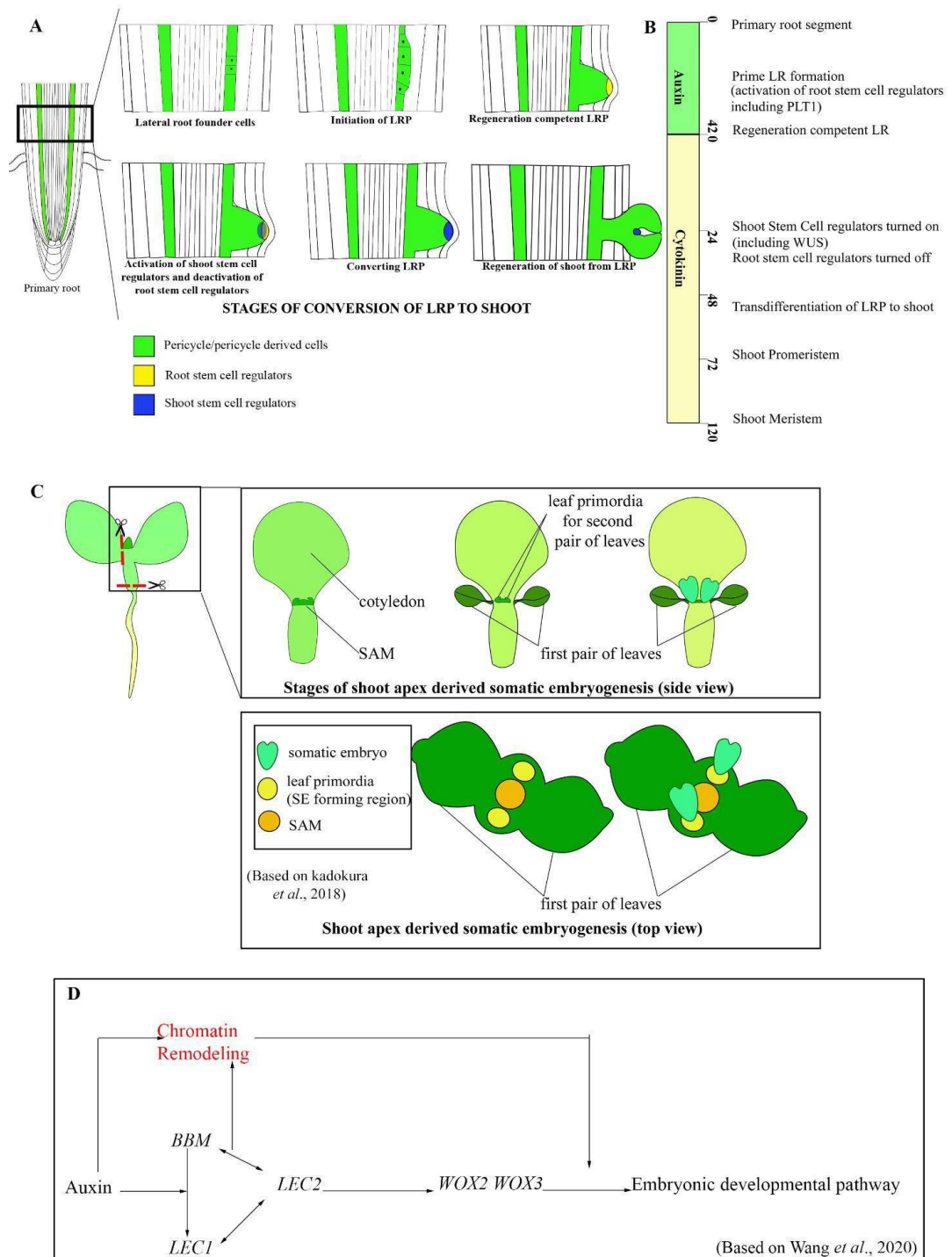


Figure. 1.3. Direct tissue culture-induced regeneration. (A) The stages involved in shoot regeneration via direct conversion of lateral root primordia (LRP). The primary root is primed to produce regeneration-competent LRP, which, when induced with shoot inductive

cues, develop into a shoot. Note that the LRP arises from cells of the pericycle. By the time shoot stem cell regulators such as WUSCHEL (WUS) are activated, root stem cell regulators such as PLT1 are rapidly deactivated and become undetectable in the converting LRP. **(B)** Timeline of the events occurring during conversion (trans-differentiation) of an LRP to a shoot. During the first stage (0-42 h; green) the primary root is incubated on auxin-rich medium, after which stage (0-120 h; yellow) the auxin-primed root is incubated on cytokinin-rich medium. **(C)** Direct regeneration of somatic embryos from leaf primordia. The developing young leaf primordia of the second pair of leaves can be induced to form a somatic embryo. **(D)** Regulatory module controlling the activation of embryonic developmental pathways during somatic embryogenesis.

Tracking a battery of cell-fate determinants in response to shoot inductive cues in real-time has provided evidence that the initial assembly of the SAM *de novo* follows a path distinct from that used for SAM development during embryogenesis (Gordon *et al.*, 2007; Sugimoto *et al.*, 2010; Kareem *et al.*, 2015). In the absence of embryonic positional cues, shoot regeneration is likely guided by an inherent property of regenerating cells to self-organize into a shoot (Gordon *et al.*, 2007; Radhakrishnan *et al.*, 2018). This self-organization is predominantly determined by two factors: acquired positional cues and the co-ordinated cellular and molecular interactions among regenerating progenitors. However, the molecular nature of callus heterogeneity and self-organization remains to be fully explored.

1.4.2. *De novo* shoot regeneration via trans-differentiation

Regeneration of a complete shoot system can also be achieved without an intermediary callus, whereby an LRP within a root explant is reprogrammed to generate a shoot directly, providing a classic example of trans-differentiation (Atta *et al.*, 2009; Chatfield *et al.*, 2013; Kareem *et al.*, 2016; Rosspopoff *et al.*, 2017). The transient expression

of root stem cell regulators and an abundance of cytokinin are key determinants for shoot regeneration via trans-differentiation. Mutants such as *plt3/5/7*, which do not express root stem cell regulators, fail to undergo LRP trans-differentiation even in the presence of abundant external cytokinin (Kareem *et al.*, 2015). Thus, the necessity of root stem cell regulators appears to be common for both callus-mediated shoot regeneration and trans-differentiation-based regeneration.

The cells within the quiescent centre (QC) of LRP are receptive to both auxin and cytokinin during a narrow developmental window, and they assume distinct fates depending on the abundance of a particular hormone (Kareem *et al.*, 2015, 2016; Rosspopoff *et al.*, 2017) (Figure. 1.3.A, 1.3.B). Although auxin treatment induces LR, cytokinin treatment of the root explants precisely during this developmental window ensures trans-differentiation to a shoot. This trans-differentiation is achieved by rapidly downregulating root stem cell regulators and turning on shoot stem cell regulators in QC cells (Figure. 1.33B) (Rosspopoff *et al.*, 2017). Thus, the QC cells in LRP are highly plastic during this narrow window. This plasticity can be exploited by repeatedly switching the explants between cytokinin and auxin treatments, thereby switching between shoot and root fates, respectively (Rosspopoff *et al.*, 2017). The multiple-rounds of cell fate switches are most likely attributed to a transient mixed-cell fate identity during the brief period of LRP development.

I.4.3. Regeneration of complete plant system via somatic embryogenesis

In addition to generating shoots and roots, explants can give rise to an entire embryo via the process of somatic embryogenesis. In this process, the somatic cells of either zygotic

tissue or young plant tissue can be induced (often with external synthetic auxin higher than that required for *de novo* shoot regeneration) to form somatic embryos. Immature zygotic tissues such as the suspensor can be cultured *in vitro* to generate somatic embryos (Wu *et al.*, 1992; Gaj, 2001; Radoeva *et al.*, 2020). Similarly, somatic embryos can be induced from adult somatic tissues such as leaf protoplasts, floral buds and the shoot apex. A well-thought notion was that the meristem from the shoot apex was the source for somatic embryos (Luo & Koop, 1997; Ikeda-Iwai *et al.*, 2003). However, careful analysis through RNA-seq and live-imaging of numerous fate determinant markers revealed that somatic embryos are induced directly from the base of young leaf primordia of the shoot apex and not the SAM (Figure. 1.3.C) (Kadokura *et al.*, 2018). Here, the leaf primordia cells are competent to produce somatic embryos only during a narrow developmental window, and leaf primordia cells outside this developmental stage die or resort to callusing (Kadokura *et al.*, 2018). Upon somatic embryo induction, the young leaf primordia cells start expressing embryogenic root identity genes as well as SAM identity genes, and thus acquire a mixed cell-fate identity. Such a molecular environment in the leaf primordia cells is likely to facilitate the switch to an embryonic fate (Kadokura *et al.*, 2018). A closer examination at the chromatin level has revealed that somatic embryogenesis involves highly organized chromatin remodelling. Using a combination of assays for transposase-accessible chromatin sequencing (ATAC-seq), RNA-seq and chromatin immunoprecipitation (ChIP)-seq, it was found that auxin modulates the chromatin accessibility and thereby the dynamics of global gene expression in a developmental stage-specific manner during somatic embryogenesis (Figure. 1.3.D) (Wang *et al.*, 2020a). Numerous TFs controlling somatic embryogenesis have also been

identified (Horstman *et al.*, 2017). For example, ectopic overexpression of a member of the AP2/ERF family of TFs, BABY BOOM (BBM; also known as PLETHORA4), which acts as a root stem cell regulator during normal development, induces somatic embryos (Boutilier *et al.*, 2002; Galinha *et al.*, 2007). BBM has been shown to activate early embryogenic genes such as WOX2 and WOX3 via the TFs LEAFY COTYLEDON 1/2 (LEC1/2), wherein BBM binds to the promoter of LEC1 and LEC2 to transcriptionally upregulate their expression (Figure. 1.3.D) (Horstman *et al.*, 2017; Wang *et al.*, 2020a). In addition to genetic control, these genes are epigenetically regulated. Concurrently, it was shown that removal of epigenetic repression marks on LEC2 favours somatic embryogenesis from root hairs (Ikeuchi *et al.*, 2015). Thus, SAM formation during somatic embryogenesis is likely to be predetermined by embryonic positional cues, unlike the callus-mediated shoot regeneration that occurs in the absence of such positional cues.

I.5. Mechanical injury-induced regeneration

I.5.1. Wound repair and regeneration in underground organs

Plants exhibit numerous mechanical injury-induced regenerative responses throughout their body parts during normal growth. Growing roots, for instance, are prone to injury by biotic and abiotic factors, often leading to loss of root cells or even the root tip. Laser ablation, cytotoxic drug treatment, the resection of *Arabidopsis* root tips, as well as simulations mimicking ablation and resection have been used to study the mechanisms underlying wound-induced regeneration (van den Berg *et al.*, 1995, 1997; Grieneisen *et al.*, 2007; Fulcher & Sablowski, 2009; Sena *et al.*, 2009; Zhang *et al.*, 2016; Hong *et al.*, 2017).

The root meristem undergoes small-scale local regeneration responses in the form of restoration of individual damaged cells, including the QC (Figure. 1.4). The targeted laser ablation of QC cells disrupts auxin flow and rapidly upregulates the auxin response a few cell layers above the damaged QC. This proximal shift in auxin responses allows vascular cells present 1-2 cell layers above the ablated QC to acquire a stem cell fate and get respecified into new QC cells, whereas cell layers between the ablated and regenerated QC get respecified into columella cells (van den Berg *et al.*, 1995; Xu *et al.*, 2006) (Figure. 1.4.E). Laser ablation of a root meristem cell other than a QC cell stimulates its inner adjacent cell to acquire a stem cell fate and undergo periclinal division. Fate re-specification of the resulting daughter cell assures restoration of the lost cell; this phenomenon stays true to all major root meristem cell types (van den Berg *et al.*, 1995; Marhava *et al.*, 2019) (Figure. 1.4.E). Positional cues have been implicated in the replacement of lost cells, and turgor pressure shock from dead cells has been suggested to influence the expansion of neighbouring cells (van den Berg *et al.*, 1995; Marhava *et al.*, 2019; Hoermayer *et al.*, 2020). However, why division and re-specification is exclusive to the cell inner to damaged ones remains elusive. It is known that growing roots experience intrinsic axial and radial growth pressure, the effect of which presumably differs between inner and outer cell files (Clark *et al.*, 2003). This differential growth pressure possibly renders the inner cell to compensate for loss of its outer cell.

Besides small-scale regenerative responses, the root meristem exhibits comparatively large-scale regeneration, for example the restoration of excised root tips. A combination of lineage tracing, single-cell RNA-seq, and live imaging has unveiled that *Arabidopsis* root

tip restoration upon resection follows an embryonic pathway (Efroni *et al.*, 2016). Here, proliferating cells at the cut end experience a brief overlap in auxin and cytokinin expression domains akin to that observed in the embryo. This similarity in hormonal dynamics triggers the expression of early embryonic genes such as MONOPTEROS and guides the subsequent activation of root stem cells, which leads to complete root tip restoration (Efroni *et al.*, 2016) (Figure. 1.4.A). Interestingly, root tip excisions close to the distal end of the root meristem allow root tip restoration, whereas proximal excisions within the root meristem fail to do so. Thus, there exists a boundary in the root meristem beyond which the root tip fails to regenerate. The dosage of the root stem cell regulator PLT2, which is expressed in a gradient along the root meristem, instructs this boundary (Figure. 1.4.C). The mitotic dilution and cell-to-cell movement of PLT2 facilitates its graded distribution from the distal to the proximal end of the root meristem; high levels of PLT2 activate stem cells, low levels promote cell division, and even lower levels are required for differentiation (Mähönen *et al.*, 2014). PLT2, together with other redundant PLT genes, is essential for root tip regeneration and acts in a positive auto-regulatory loop to grant regeneration potential (Figure. 1.4.D). This auto-regulatory loop collapses upon sustained overexpression of PLT2. In such conditions, the root meristem of uninjured roots grows longer than normal, but its root tip restoration ability ceases and gets substituted with mere residual cell proliferation (Figure. 1.4.D). Thus, the threshold-sensitive auto-regulatory loop distinguishes the growth of the organ from its regeneration potential (Durgaprasad *et al.*, 2019).

Auxin biosynthesis and its polar transport are pivotal for organ formation during normal development as well as for root tip regeneration (reviewed by (Shanmukhan and

Mathew *et al.*, 2020). The extent of injury, i.e. large-scale or small-scale, acts as the decisive factor for the magnitude of auxin required for cell or organ regeneration. In the case of minor injuries to the root meristem, such as targeted cell damage, ongoing auxin production and its accumulation due to impeded transport is sufficient to stimulate small-scale regeneration (Canher *et al.*, 2020). However, large-scale regeneration such as root tip restoration necessitates further upregulated auxin production (Matosevich *et al.*, 2020). Several root fate determinants and regulators of root stem cell maintenance have been implicated in regeneration, although how these regulators function in conjunction with auxin needs to be studied (Xu *et al.*, 2006; Efroni *et al.*, 2016; Marhava *et al.*, 2019; Durgaprasad *et al.*, 2019; Matosevich *et al.*, 2020). An interesting possibility is that the fate determinants and auxin act in a regulatory loop to control the regeneration of specific cell types or organs.

Auxin, along with another plant hormone jasmonic acid (JA), triggers a common signalling network to accomplish regeneration following root tip excision as well as ablation. The two hormones accumulate locally upon injury and act synergistically to trigger expression of the stress response protein ETHYLENE RESPONSE FACTOR 115 (ERF115) and a cell cycle regulator CYCLIN D6;1 (CYCD6;1), which in turn activates the RETINOBLASTOMA-RELATED (RBR)-SCR-SHORTROOT (SHR) protein network (Zhou *et al.*, 2019) (Figure. 1.4.B). Root tip restoration necessitates reactivation of the SCN, which in turn is regulated by the RBR-SCR-SHR protein network (Zhou *et al.*, 2019). The variety of regenerative responses in the Arabidopsis root meristem discussed here relies on regulators that are required not only for regeneration, but also for root meristem development, thereby providing a nice example of the interplay between regenerative

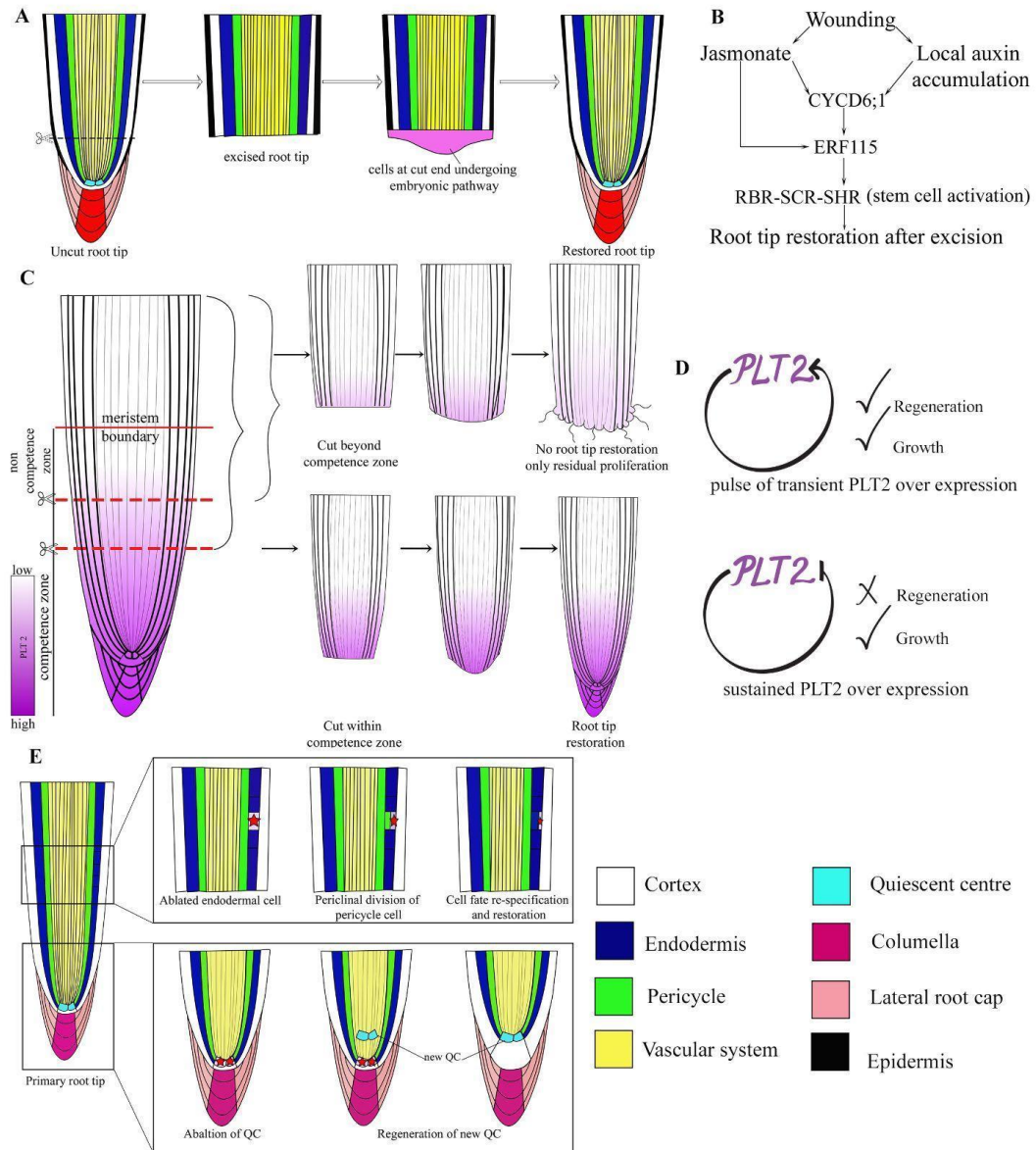


Figure. 1.4. Regeneration of the root tip and its cells. (A) Upon root tip excision, uninjured endodermal/stelar cells at the cut end proliferate and undergo transient activation of embryonic signalling pathways to restore the missing root tip. (B) Regulatory module showing how wound-induced jasmonic acid (JA) and auxin accumulate to trigger stem cell activation (i.e. the RBR-SCR-SHR protein network) via the ethylene response gene, ERF115, and a regulator of cell cycle progression, CYCLIN D6 (CYCD6;1). This ultimately enables root tip restoration following excision and stem cell niche regeneration after quiescent centre (QC) ablation. (C) The concentration of PLETHORA 2 (PLT2), a transcription factor that is expressed in a gradient along the root meristem, instructs the boundary for root tip restoration. The root tip is restored when excision is within the region

of meristem expressing high levels of PLT2 (top) but restoration fails when the tip is excised in a region expressing low PLT2 levels (bottom). **(D)** Schematic depicting the importance of the PLT2-autoregulatory loop in regeneration and how it distinguishes growth of the root meristem from its regeneration potential. **(E)** Upon ablation of an endodermal cell (top inset), a pericycle cell from the inner cell file undergoes periclinal division and the resulting daughter cell respecifies its fate to replace the lost endodermal cell. Targeted laser ablation of the QC (bottom inset) results in its regeneration two cell layers above the initial QC, while the cell layers below the new QC become specified into columella cells.

mechanisms and those governing development (Hardtke & Berleth, 1998; Galinha *et al.*, 2007; Cruz-Ramírez *et al.*, 2012, 2013; Mähönen *et al.*, 2014; Efroni *et al.*, 2016; Zhou *et al.*, 2019; Durgaprasad *et al.*, 2019).

Auxin also plays a role in root regeneration following abiotic stress, for example following exposure to cold temperatures. Here, cells of the SCN – including QC cells – are protected by sacrificing columella stem cell daughters (CSCDs). Similar to the anatomical block in auxin transport by ablated QC cells, which leads to regeneration of new QC, the dead CSCD cells block auxin transport resulting in elevated auxin levels in the QC, which in turn prolongs the existing QC instead of regenerating a new one (Hong *et al.*, 2017). Other multi-functional plant growth hormones such as ethylene, mostly known for its role in fruit ripening and senescence, are also implicated in the regenerative response to stress. For example, biotic stress such as a nematode attack in the root meristem (which can be considered analogous to laser ablation) stimulates ethylene signalling in addition to JA signalling. Whereas ethylene elicits a local defense response against the nematode, JA encourages its reproductive success besides promoting root regeneration (Zhou *et al.*, 2019). These two pathways, which are seemingly antagonistic to each other, are likely to promote plant vitality through defense and regeneration. Studies addressing the fundamental mechanisms of root regeneration and their physiological relevance in *Arabidopsis* therefore open up the possibility of carrying out similar such studies in other plant species. This will

also allow parallels between parasite-induced responses and mechanical-injury induced responses to be drawn.

I.5.2. Wound repair and vascular regeneration in aerial organs

Much like underground parts, aerial parts of the plant are injured frequently and elicit varying regenerative responses. Injuries mimicking natural mechanical damages to growing *Arabidopsis* aerial organs, such as vascular injury and surface abrasion, have allowed the molecular mechanisms underlying their regeneration to be probed. Both surface abrasion and deep incisions in the stem that slice through the vasculature trigger cell proliferation; however, subsequent vascular regeneration and reunion is exclusive to the latter (Figure. 1.5.A) (Flaishman *et al.*, 2003; Asahina *et al.*, 2011; Radhakrishnan *et al.*, 2020a). During vascular reconnection, the regenerating vasculature bypasses the wound and this phenomenon is observed following stem as well as leaf mid-vein incisions (Figure. 1.5.A, 1.5.B) (Flaishman *et al.*, 2003; Radhakrishnan *et al.*, 2020a). The presumption that ground tissue can be induced to form vascular cells during vascular regeneration in the presence of sufficient auxin flux led to the auxin canalization hypothesis (Sachs, 1969, 1981, 1991). Auxin canalisation is promoted by polar localization of phosphorylated PIN1 (an auxin efflux carrier) on the plasma membrane during vascular regeneration in the *Arabidopsis* stem (Hajný *et al.*, 2020). PIN1 fails to polarise in the absence of a TF-based regulatory axis (discussed below) leading to failure of vascular regeneration in growing leaves and further substantiating the canalization model for vascular regeneration (Radhakrishnan *et al.*, 2020a).

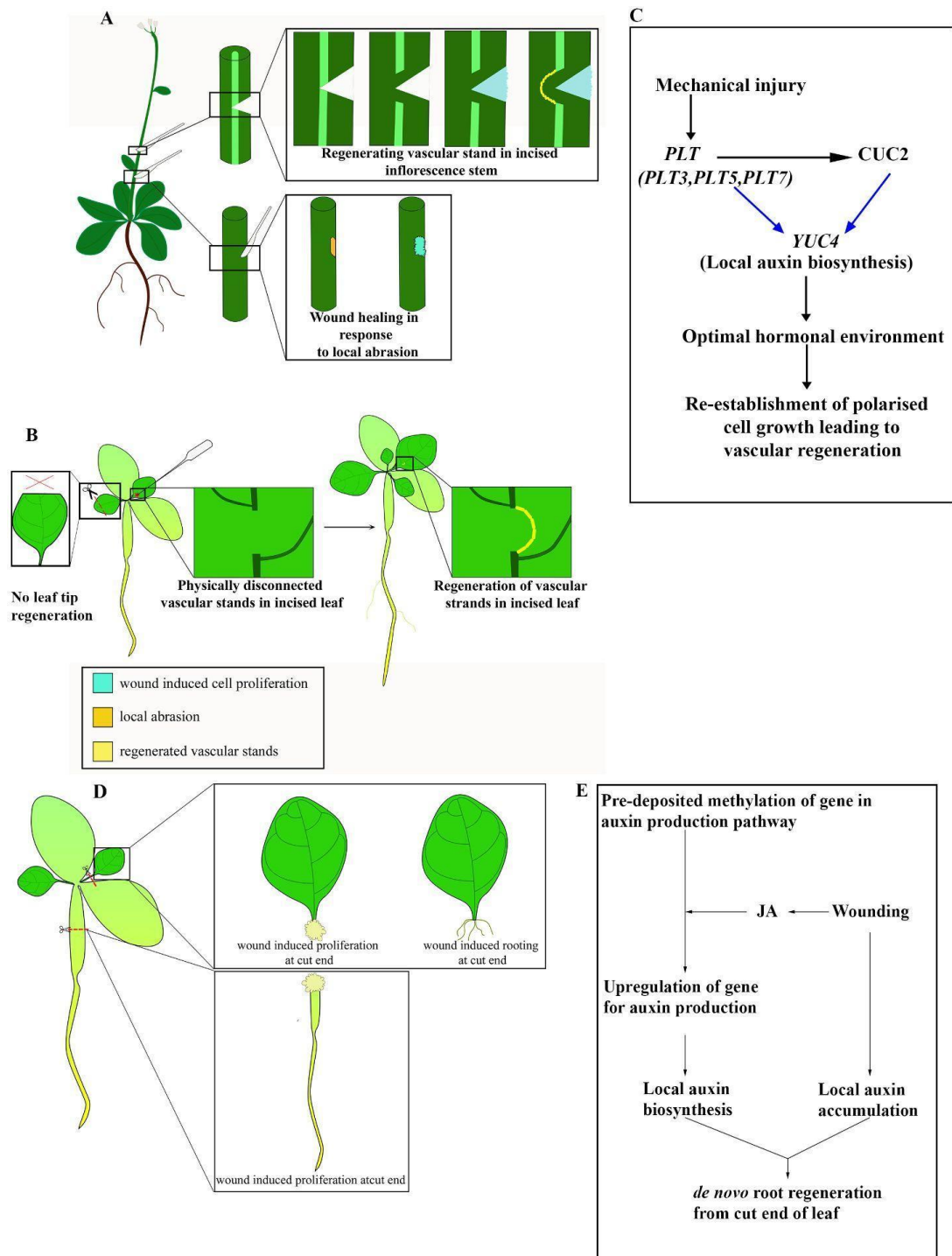


Figure. 1.5. Regenerative responses in *Arabidopsis* aerial organs. (A) Incision of the inflorescence stem (top inset) disrupts vascular tissue continuity and induces cell proliferation to seal the gap between the disconnected tissues. Subsequently, vascular continuity is reinstated by the vascular strands regenerating around the wounded area. By contrast, surface abrasion of the inflorescence stem (bottom inset) induces only local cell

proliferation to heal the injury. **(B)** Excision of the distal end of a growing leaf (left inset) does not initiate regeneration to replace the lost part. By contrast, incision of the leaf mid vein (right inset) disrupts vascular continuity. The regenerating vasculature circumvents the wounded area to re-establish vascular continuity in the growing leaf. **(C)** Schematic depicting the PLT-CUC2 regulatory module involved in vascular regeneration in aerial organs. **(D)** A regenerative response occurs (in the form of wound-induced callus formation) from the cut end of a detached leaf when the cut end does not touch the media (top left). However, when the cut end of the detached leaf is in contact with the nutrient-rich media, wound-induced rooting occurs (top right). Wound-induced callusing also occurs at the cut end of detached hypocotyls when placed onto nutrient-rich media (bottom). **(E)** Regulatory module showing the mechanism of de novo root regeneration from the cut end of a detached leaf.

Vascular regeneration in growing *Arabidopsis* aerial organs requires the activity of PLT3/5/7 and AINTEGUMENTA genes. However, in this context, PLT3/5/7 do not activate root stem cell regulators like they do in tissue culture-mediated regeneration, in which their activation is indispensable. Instead, PLT genes directly bind to the promoter of another TF gene, CUC2, in response to injury. The PLT-CUC2 regulatory axis acts in a coherent feedforward loop to upregulate local auxin production, which in turn is essential for vascular regeneration and for guiding the newly formed vein along a polarized path for reunion with its parental strands (Figure. 1.5.C) (Radhakrishnan *et al.*, 2020a). Here, the high auxin signalling is likely to serve as a cue for ground tissue cells to undergo vascular cell activation that appears to steer the path of the regenerating vein. The likely termination of this feed-forward loop could thus be causal for the cessation of vein regeneration upon complete reconnection between parental strands.

Nevertheless, how the regenerating vein follows a path and recognizes its parental strands hitherto remains unknown. Tracking the path of this injury-induced newly formed vein offers an excellent system to study the recognition, communication and reunion between physically disconnected tissues. In addition, vein regeneration efficiency declines progressively with increasing seedling age, with the increase in wound size, and as the position of injury shifts farther from the leaf-base (Radhakrishnan *et al.*, 2020a,b). Although

the exact mechanism underlying the age- and position-dependent efficiency of this regeneration is unknown, it could likely be attributed to modulation of the PLT-CUC2 regulatory axis. Strikingly, vein development and patterning in *plt* or *cuc2* mutants remain unaffected during normal growth (Radhakrishnan *et al.*, 2020a). The injury-induced PLT-CUC2 regulatory axis therefore distinguishes vein regeneration from its formation during normal development.

I.5.3. Regenerative responses at the cut ends of detached organs: insights into plasticity and ageing

A feature unique to plant regeneration is the ability of cells at the cut end of a detached organ to adopt an identity different from their pre-existing one. For example, when cultured on hormone-free medium, an excised *Arabidopsis* leaf elicits one of the two following regenerative responses from its cut end: the formation of a new organ, i.e. *de novo* root regeneration (DNRR), or local healing in the form of callus formation (Figure. 1.5.D) (Iwase *et al.*, 2011, 2017; Chen *et al.*, 2014; Ikeuchi *et al.*, 2016).

During DNRR, the rapid yet transient accumulation of wound-induced JA, in conjunction with pre-deposited methylation on JA-responsive genes, upregulates auxin production at the site of injury, which in turn facilitates DNRR (Figure. 1.5.E) (Zhou *et al.*, 2019). The auxin maxima precede vascular cell proliferation and the induction of WOX genes, namely WOX11 and WOX12 near the cut end of the leaf, eventually resulting in the formation of a small mass of cells with rooting competence (Bustillo-Avendaño *et al.*, 2018; Zhou *et al.*, 2019). Such cells undergo a two-step cell fate transition to initiate the formation

of a root primordia: WOX11/12 along with LBD genes regulates the transition of rooting competent cells into root founder cells, marking the first step, whereas WOX5/7 regulate the transition of root founder cells into a root primordium, marking the second step (Liu *et al.*, 2014; Hu & Xu, 2016). The subsequent formation and maintenance of the root primordium by root stem cell regulators results in activation of the root meristem and, ultimately, root regeneration (Bustillo-Avendaño *et al.*, 2018). In addition to these TFs, PLT proteins are essential for DNRR (Shanmukhan *et al.*, 2021). Moreover, a decline in this form of root regeneration has been observed from aged leaf explants. Although the implications of ageing on many aspects of development have been well documented in both plants and animals, this area remains less explored with respect to regeneration. At least in DNRR, the diminished regeneration potential of older *Arabidopsis* leaf explants is partly attributed to the age-induced accumulation of ethylene, which inhibits WOX genes and, consequently, DNRR (Li *et al.*, 2020).

Aside from DNRR, the cut end of detached leaf exhibits a distinct wound-induced callusing response. Here, the activation of an early wound response gene, WOUND INDUCED DEDIFFERENTIATION 1 (WIND1), causes cells at the cut end to undergo dedifferentiation and subsequent cell proliferation to form a callus (Figure. 1.5.E) (Iwase *et al.*, 2011). Unlike the hormone-induced callus that arises in tissue culture, the wound-induced callus does not predominantly express root fate determinants (Iwase *et al.*, 2011). In addition, the hormone-induced callus arises from XPP cells and spreads throughout the explant, whereas the injury-induced callus forms from and is confined to the site of injury (Atta *et al.*, 2009). This is because the regeneration machinery distinguishes the uninjured

tissues from the injured ones by tight transcriptional control of re-specification genes. Such control is mediated partly by epigenetic mechanisms, whereby modifications to histone N-terminal tails regulate transcription of the associated genes. For example, the majority of genes that are rapidly induced by wounding, including WIND1, are marked by histone acetylation immediately before/after wounding, thereby allowing them to promote callus formation only at the site of injury. A correlation between the expression of wound-responsive genes and their epigenetic modifications has recently been revealed using ChIP-seq and RNA-seq (Rymen *et al.*, 2019).

The deciding factor for either of the two responses – DNRR or callus formation – at the cut end of a detached leaf remains unclear. DNRR results when the cut end touches the media, as opposed to callus formation, which occurs when the cut end fails to touch the media (Figure. 1.5.D) (Iwase *et al.*, 2017; Shanmukhan *et al.*, 2021). As an increase in local auxin production is essential for both these responses, what distinguishes DNRR from callus formation is likely a touch-dependent mechanical cue (Iwase *et al.*, 2017; Shanmukhan *et al.*, 2021). Among the several possible mechanical cues, osmotic stress has been implicated in the regeneration of root meristem cells upon targeted cell ablation (Hoermayer *et al.*, 2020). However, the mechanisms underlying touch-driven mechano-sensing in regeneration remain largely unknown. Presumably, in DNRR at least, contact of the cut end with media activates touch-dependent mechano-sensors, and the resulting signal transduction in conjunction with local auxin accumulation likely promotes the production of rooting-competent cells (Shanmukhan *et al.*, 2021).

I.5.4. Grafting: fusion between two body parts

The age-old agricultural technique by which two different body parts originating from two plants of the same or different species are joined to give rise to a chimera is called grafting. The parts that form the prospective shoot and root systems in a graft are called the scion and rootstock, respectively. Initially, grafting was practiced to combine aesthetically and agronomically desirable characteristics from two plants, such as disease resistance, increased productivity, and early flowering (Goldschmidt, 2014). However, grafting in *Arabidopsis* has now become more relevant for addressing fundamental questions concerning the long-range transport of proteins, hormones, RNAs, and secondary metabolites. Importantly, although it is a type of mechanical injury-induced regeneration, grafting by itself is a distinctive phenomenon and, as such, can provide insights into the molecular mechanisms involved in regeneration. Refined techniques have allowed micrografting of hypocotyls, leaves, and cotyledons in *Arabidopsis* (Nisar *et al.*, 2012; Yoo *et al.*, 2013; Huang & Yu, 2015; Bartusch *et al.*, 2020). Such studies have unveiled the sequential events involved in grafting, from recognition of disrupted tissue continuity to successful graft formation.

Micrografting of *Arabidopsis* hypocotyls has revealed that, after cutting, auxin and starch accumulate above the cut and their asymmetric distribution on either side of the cut indicates a disruption in tissue continuity (Melnyk *et al.*, 2018). In order to re-establish tissue continuity, recognition and adhesion between the two opposing body parts is imperative. Adhesion during grafting is facilitated by a cell wall remodelling enzyme, which often functions when the opposing parts are recognized as compatible (Notaguchi *et al.*, 2020). The cues that trigger the recognition phenomenon are less known. However, there is

evidence to suggest that the two opposing tissues have been recognized by each other. For example, the activation of HIGH CAMBIAL ACTIVITY 2 (HCA2), an auxin response gene that is required for phloem reconnection, exclusively in the grafted bottom suggests that recognition has occurred. Neither the unattached bottom nor the intact hypocotyls express HCA2, suggesting that it is uniquely activated by grafting and highlighting that phloem reconnection is crucial to initiate the re-establishment of tissue continuity (Melnik *et al.*, 2015, 2018; Matsuoka *et al.*, 2016). Another regulator of phloem reconnection is the lateral root formation gene ALF4, activity of which is required below the graft junction (DiDonato *et al.*, 2004; Sugimoto *et al.*, 2010; Melnyk *et al.*, 2015). Sealing of the wound gap and subsequent vascular regeneration are common events in both partially incised stems of the same plant as well as in grafting between two plants, but what distinguishes grafting is the recognition upon tissue attachment (Figure. 1.6.A, 1.6.B) (Melnik *et al.*, 2018; Radhakrishnan *et al.*, 2020a).

Refined techniques have been developed over the last decade to detect, monitor, and quantify the movement of molecules across the graft junction. For example, the application of carboxyfluorescein diacetate (a membrane permeable fluorescent dye) to the scion and rootstock can be used to assay phloem and xylem connectivity, respectively (Melnik *et al.*, 2015).

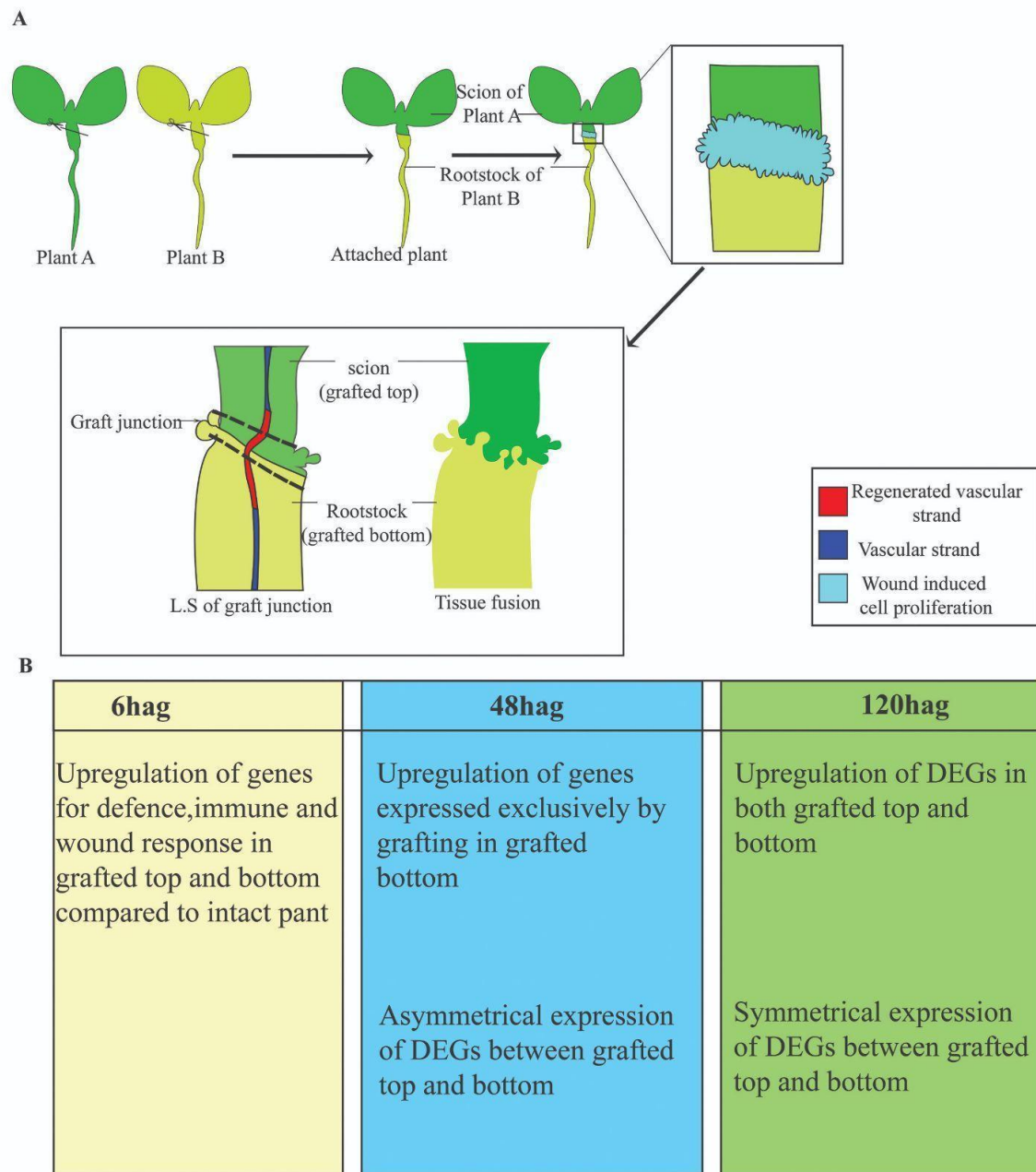


Figure. 1.6. Grafting between two plant parts. (A) The scion from plant A and the rootstock from plant B are attached together, resulting in wound-induced cell proliferation in the form of a callus (see inset). This seals the gap at the attachment plane and forms a graft junction (indicated by dashed lines). Subsequently, cells from both opposing parts interdigitate, ultimately leading to tissue fusion and re-establishment of vascular continuity. Note that the epidermal cells of a grafted top and bottom expand at the graft junction. (B) Timeline over which differentially expressed genes (DEGs) shift from being asymmetrically to symmetrically distributed between the grafted top and bottom. Note that DEGs are genes

that are differentially expressed in grafted seedlings compared with intact seedlings. hag, hours after grafting; L.S., longitudinal section.

In addition, live imaging using confocal microscopy, qRT-PCR, and RNA-seq of transgenics and mutants has yielded reliable data regarding long-range transport of mobile molecules in *Arabidopsis* (Turnbull, 2010; Melnyk *et al.*, 2015, 2018). Although challenging, understanding the role of these long-range mobile molecules for plant regeneration will contribute significantly to future studies in the field.

I.6. DISCUSSION

The advances in tools and resources available for *Arabidopsis* research have begun to provide deeper insights into the journey of plant regeneration and offer a promising direction for future studies in the area of cellular reprogramming and developmental plasticity. Auxin plays a crucial part in several cellular processes, including cell fate transitions during both development and regeneration, acting via global genetic regulation and epigenetic modifications, including chromatin remodeling. It functions in a concentration-dependent manner to yield a particular developmental outcome. As such, each regenerative response may depend on a specific auxin level. How a specific level of auxin is assigned to, and controls, a particular regenerative response as well as normal development can likely be attributed to: (1) context-dependent factors; (2) the magnitude of auxin production; (3) the source of auxin production; (4) its diffusion; (5) its polar auxin transport which generates a flux.

The generation of specific auxin levels during normal development has been explained *in silico* using two key models: the reaction-diffusion (RD) model and the flux-based model (Mündermann *et al.*, 2005; Smith *et al.*, 2006; Grieneisen *et al.*, 2007; Cruz-Ramírez *et al.*, 2012; Sampathkumar *et al.*, 2014). The flux-based auxin canalization model elegantly explains gradient-driven self-organizing patterning (Sachs, 1969, 1981, 1991; Bennett *et al.*, 2014). However, the model fails to sustain itself after a temporal perturbation, and a major limitation is to test the model experimentally, particularly to measure flux (van Berkel *et al.*, 2013). As such, both the RD and flux models are likely required to explain the complex cell-cell interactions, and thereby the emergence of pattern, during regeneration. The expanse of data derived from experimental studies in *Arabidopsis* can now be used to examine the possibility of a switch between the two models, and how such a switch occurs during different regenerative responses.

Plants must be equipped to ensure the correct replacement of an organ or tissue lost in injury. As an immediate response to injury, cells at the wound vicinity experience degrees of variation with respect to noise in gene expression, cell polarity, and cell division state, the cumulative effect of which is harnessed for the emergence of a particular pattern. However, not all such variations will be reflected in cellular behavior. Thus, it will be challenging to tease out only the meaningful variations to interpret cellular heterogeneity. Although it will be daunting, finding the meaningful variations and precisely quantifying them will be key to gaining deeper insights into cellular reprogramming and fate transitions during regeneration. Moreover, when plants encounter injuries in varying conditions of nutrient availability and environmental stimuli, such as electrical stimuli, nitric oxide, and physical contact, they are

likely to tweak their innate wound repair mechanism in response to these conditions (Kral *et al.*, 2016; Cervantes-Pérez *et al.*, 2020; Shanmukhan *et al.*, 2021). The challenge will be to test how fluctuating environmental conditions modulate regeneration-specific regulatory frameworks and to understand their physiological relevance. Such a study, already carried out in the *Arabidopsis* root tip (Marhavý *et al.*, 2019), offers encouragement to conduct the same study in other plant parts and in other species in order to better understand how plants sense and respond to injury.

Chapter II: Materials and method

This work is a part of the published manuscript:

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Protocol for real-time imaging, polar protein quantification, and targeted laser ablation of
regenerating shoot progenitors in Arabidopsis. ***STAR protocols***, 4(2), 102184.

<https://doi.org/10.1016/j.xpro.2023.102184>

(*Co-corresponding author)

"What we observe is not nature itself, but nature exposed to our method of questioning."
— Werner Heisenberg

II.1. MATERIAL DETAILS

Table 2.1: Oligonucleotides (Genotyping primers)

OLIGONUCLEOTIDE NAME	5`-3`
AT1G02360-LP	ACGCCATTGATGATCAACTTC
AT1G02360-RP	GGTCCAATTCAACTATCGTGG
EXPB1-LP	AACTTTGATGCAATGTCTGGG
EXPB1-RP	TCAAATTTGGATGCGTTTAGG
AT5G48900-LP	ACTACACGAGGGAGATGCATG
AT5G48900-RP	CATTACTTGACGTTTTGGTTG
EXPA10-LP	AACTGGTTACATAACACGCACG
EXPA10-RP	CCAAGATGACCCATGTTTACG
EXPA1-LP	ACACGACTTCTTCATCGATCG
EXPA1-RP	CGTAACCATTGACATGTGACG
EXPA8-LP	ACCTTGCCATACAAATTTCC
EXPA8-RP	TTCAAAGTAAATTGCCCCGTTG
BS1-LP	TCTCTCTCTTCTTTTCCGGC
BS1-RP	TCTTGACGATTTAGGGAACC

IAA30-LP	CGGAACAATTGTAATATCTCCG
IAA30-RP	AGGGAGAAGCTCATCGTCTTC
HSL2-LP	CGTCTTGAGCTAGCCAACAAC
HSL2-RP	AAATCGTGTCGTCCAATCAAG
SNRK2.3-LP	AATATTGTAATTGGGCTGGCC
SNRK2.3-RP	AACCTTGTCTCCTCTCTCGATG
RGF3-LP	TCATATGTAGAAGCTGGCATGC
RGF3-RP	GAATATGGACGTTATGGCAGG
PHB-LP	TTCAGATGATTGGGATGAAGG
PHB-RP	TAACAGCATCATTGAAACCCC
AN3-LP	TTCCTTGTGCCCACTCTTTC
AN3-RP	TGTGAATATTGTGGGTGGTCC
FEZ-LP	TAGACATTGTGTCCGGTGCTTG
FEZ-RP	GTATAGGAACAGCTCGAGGCC

Table 2.2: Experimental models: Organisms/strains

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Arabidopsis thaliana:</i> <i>Columbia</i> ;pPIN1::gPIN1:GFP (7-dpg)	(Gordon et al., 2007; Kareem et al., 2015)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> ;pXTH9::gXTH9:YFP, pPIN1::gPIN1:GFP (7-dpg)	(Varapparambath and Mathew et al., 2022)	N/A

<i>Arabidopsis thaliana</i> <i>Columbia</i>	(Kareem et al., 2015)	N/A
<i>xth9</i> (insertion in 3'UTR)	(Xu & Cai, 2019)	N/A
<i>cuc2-3</i>	(Hibara et al., 2006)	N/A
<i>cuc1-5</i>	(Hibara et al., 2006)	N/A
<i>cuc1-5,cuc2-3</i>	(Hibara et al., 2006)	N/A
<i>Arabidopsis thaliana</i> : <i>Columbia</i> <i>pSOK2::gSOK2:vYFP</i>	(Yoshida et al., 2019)	N/A
<i>Arabidopsis thaliana</i> : <i>Columbia</i> <i>pSOK5::gSOK5:vYFP</i>	(Yoshida et al., 2019)	N/A
<i>Arabidopsis thaliana</i> : <i>Columbia</i> <i>pG1090:XVE::PINOID:vYFP</i>	Dr Ari Pekka Mähönen	N/A
<i>Arabidopsis thaliana</i> : <i>Columbia</i> <i>pXTH9::gXTH9:vYFP</i> in <i>pPIN1::gPIN1:GFP</i>	(Varapparambath and Mathew et al., 2022)	N/A
<i>Arabidopsis thaliana</i> : <i>Columbia</i> <i>pCLASP::vYFP-gCLASP:3AT</i> in <i>pPIN1::gPIN1:GFP</i>	(Varapparambath and Mathew et al., 2022)	N/A
<i>Arabidopsis thaliana</i> : <i>Columbia</i>	(Varapparambath and Mathew et al., 2022)	N/A

<i>pCUC2::gCUC2:vYFP</i> in <i>pPIN1::gPIN1:GFP</i>		
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pCYCB1;1::CYCB1;1-vYFP</i> in <i>pPIN1::gPIN1:GFP</i>	(Varapparambath and Mathew <i>et al.</i> , 2022)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pWUS::erCFP:tWUS</i> in <i>plt3,plt5-2,plt7;</i>	(Varapparambath and Mathew <i>et al.</i> , 2022)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pSOK2::gSOK2:vYFP</i> in <i>pPIN1::gPIN1:GFP</i>	(Varapparambath and Mathew <i>et al.</i> , 2022)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pSOK5::gSOK5:vYFP</i> in <i>pPIN1::gPIN1:GFP</i>	(Varapparambath and Mathew <i>et al.</i> , 2022)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pWUS::3XVENUS-tWUS</i> in <i>pPIN1::gPIN1:GFP</i>	(Varapparambath and Mathew <i>et al.</i> , 2022)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pHTR2::CDT1a-vYFP, PIN1-</i> <i>GFP</i>	(Varapparambath and Mathew <i>et al.</i> , 2022)	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>pUBQ10::mCITRINE-</i> <i>KA1^{MARK1}</i>	Prof. Jvon Jaillais	NASC# N2107345

<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>xxt1, xxt2</i>	Prof. Joris Sprakel	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>feronia-4</i>	Prof. Joris Sprakel	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>katanin-1</i>	Prof. Joris Sprakel	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>fimbrin4-2, fimbrin5-2</i>	Prof. Joris Sprakel	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>fimbrin9-1, fimbrin5-2</i>	Prof. Joris Sprakel	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>sok2</i>	Prof. Dolf Weijers	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>sok1/2/3/4 quadruple mutant</i>	Prof. Dolf Weijers	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>expb-1</i>	ABRC	N/A

<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>expa-10</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>exp-8</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>bs-1</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>iaa-30</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>hsl-2</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>snrk2.3</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>rgf3</i>	ABRC	N/A

<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>phb</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>an-3</i>	ABRC	N/A
<i>Arabidopsis thaliana:</i> <i>Columbia</i> <i>fez</i>	ABRC	N/A

Table 2.3: Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige & Skoog Basal Salts Medium	Sigma	Cat# M5524
Agar Plant cell culture tested, powder	Sigma	Cat# A7921
MES hydrate	Sigma	Cat# M2933
Gamborg's B5 basal salt mixture	Sigma	Cat# G5768
Gamborg's vitamin solution 1000X	Sigma	Cat# G1019

Sucrose	Sigma	Cat# S0389
D-Glucose	Sigma	Cat# G7021
2,4-Dichlorophenoxyacetic acid (2,4-D)	Sigma	Cat# 49083
Kinetin	Sigma	Cat# K3378
Trans-zeatin	Sigma	Cat# Z0876
Biotin	Sigma	Cat# B4639
Propidium iodide (PI)	Sigma	Cat# P4170
Ampicillin	Sigma	Cat# A0166
Sodium Hydroxide (NaOH)	Himedia	Cat# TC460M
4% Sodium hypochlorite (NaClO)	Corclear	N/A
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D5879
N ⁺ BODIPY	(Michels <i>et al.</i> , 2020)	N/A
CarboTag-BODIPY	(Besten <i>et al.</i> , 2024)	N/A
Absolute Ethanol	Jebsen & Jessen (GmbH & Co.)	Cat# 58051 LO5
D-Mannitol	Sigma	Cat# M9647
Methanol	Merck	Cat# 67-56-1

Software and algorithms		
FIJI/ImageJ	(Schneider <i>et al.</i> , 2012)	https://imagej.net/software/fiji/
ZEN image processing software	Zeiss	https://www.micro-shop.zeiss.com/en/de/softwarefinder/software-categories/zen-black/zen-black-system/
Adobe Photoshop CS6	Adobe Acrobat	N/A
Adobe Illustrator	Adobe Acrobat	N/A
Excel	Microsoft	N/A
GraphPad Prism version 9.5.1	N/A	graphstats.net/prism
MorphoGraphX 2.0	(Barbier de Reuille <i>et al.</i> , 2015)	N/A
SPCImage 8.5	Prof. Joris Sprakel	N/A
Shiny GO	N/A	https://bioinformatics.sdstate.edu/go/
Instruments		
Arabidopsis Growth chamber	Percival	AR-100L3
Laminar Air Flow	Microfilt	N/A
Refrigerator	Whirlpool	FF2-550 WLP 1G2211
Sterile Disposable Square petri-dish (120 mm × 120 mm)	Himedia	PW050

Sterile Disposable Round petri-dish (35 mm)	Tarsons	460035
Micro centrifuge	Eppendorf	Centrifuge 5420
Micro centrifuge tubes (1.5 mL)	Tarsons	500010
PhytaWrap (2 inch)	Himedia	PLA002
10 mL Syringes with needle	Dispovan	13484
Syringe driven filter (45 µm pore size)	Whatman Uniflo	9914-2504
Vanna's micro-scissors (straight)	Vanna's	AGT5223
Forceps	Dumont	Style 35A
Round Glass petri-dish (150 mm × 25 mm)	Riviera	23755 52
Leica SP8-DIVE multiphoton	Leica	N/A
Zeiss LSM 880	Zeiss	N/A
ZEN2.3 SP1 black edition	Zeiss	N/A
Universal mounting frame A-K (D) - frame size 160x116mm	Zeiss	410800-1000-000
40X Water-dipping Objective (W "Plan-Apochromat" 40x/1.0 DIC M27)	Zeiss	421462-9900

Table 2.4: Gene Accession Numbers

Gene Name	Locus ID
CUC2	AT5G53950
XTH9	AT4G03210
PIN1	AT1G73590
CYCB1;1	AT4G37490
WUS	AT2G17950
SOK2	AT5G10150
SOK5	AT5G59790
PLT2	AT1G51190
CLASP	AT2G20190
CDT1-A	AT2G31270
FERONIA	AT3G51550
KATANIN	AT1G80350
XXT1	AT3G62720
XXT2	AT4G02500
FIMBRIN 5	AT5G35700
EXPB1	AT2G20750
BS1	AT1G74500
IAA30	AT3G62100
SNRK2.3	AT5G66880
RGF3	AT2G04025
FEZ	AT1G26870
BAS1	AT2G26710

PHB	AT2G34710
HSL-2	AT5G65710
EXP-8	AT2G40610
BAS-1	AT2G26710
AN3	AT5G28640

II.2. RESOURCE AVAILABILITY

II.2.1. Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Kalika Prasad (kalika.prasad@iiserpune.ac.in).

II.2.2. Materials Availability

Plasmids and transgenic plant lines generated in this study will be made available on request to the lead contact. This study did not generate new unique reagents.

II.2.3. Data and Code Availability

The RNA-Seq data will be made available on request to the lead contact.

II.3. PREPARATION OF REAGENTS, MEDIA AND SETTING UP OF INSTRUMENTS

II.3.1. preparation of reagents and media

Table 2.5: Ampicillin stock

Reagent	Final concentration (mg/mL)	Amount
Ampicillin	100 mg/mL	10 g
Absolute Ethanol	n/a	40 mL
Autoclaved Milli-Q water	n/a	60 mL
Total		100 mL

Ampicillin was dissolved in 40% ethanol to prepare the stock solution. The Ampicillin stock was then sterilized by passing it through a syringe-driven filter before being aliquoted into 1.5 mL microcentrifuge tubes.

Table 2.6: 2,4-Dichlorophenoxyacetic acid (2,4-D) stock

Reagent	Final concentration (mg/mL)	Amount
2,4-Dichlorophenoxyacetic acid (2,4-D)	1 mg/mL	100 mg
Absolute Ethanol	n/a	95 mL
Autoclaved Milli-Q water	n/a	5 mL
Total		100 mL

2,4-Dichlorophenoxyacetic acid was dissolved in 95% ethanol to prepare the stock solution. The 2,4-D stock was then sterilized by passing it through a syringe-driven filter before being aliquoted into 1.5 mL microcentrifuge tubes.

Table 2.7: Kinetin stock

Reagent	Final concentration (mg/mL)	Amount
Kinetin	1 mg/mL	100 mg
NaOH	40 mg/mL	4 g
Autoclaved Milli-Q water	n/a	100 mL
Total		100mL

Kinetin was dissolved in 1N NaOH to prepare a 1 mg/mL stock solution. The Kinetin stock was then sterilized by passing it through a syringe-driven filter before being aliquoted into 1.5 mL microcentrifuge tubes.

Table 2.8: d-Biotin stock

Reagent	Final concentration (mg/mL)	Amount
d-Biotin	10 mg/mL	1 g
NaOH	40 mg/mL	4 g
Autoclaved Milli-Q water	n/a	100 mL
Total		100 mL

d-Biotin was dissolved in 1N NaOH to prepare a 10 mg/mL stock solution. The d-Biotin stock was then sterilized by passing it through a syringe-driven filter before being aliquoted into 1.5 mL microcentrifuge tubes. The 1 mg/mL d-Biotin stock was stored at -20°C until use.

Table 2.9: Trans-zeatin stock

Reagent	Final concentration (mg/mL)	Amount
Trans-zeatin	1 mg/mL	100 mg
DMSO	n/a	100 mL
Total		100 mL

Trans-zeatin was dissolved in DMSO to prepare a 1 mg/mL stock solution. The Trans-zeatin stock was then sterilized by passing it through a syringe-driven filter before being aliquoted into 1.5 mL microcentrifuge tubes.

Table 2.10: Propidium Iodide

Reagent	Final concentration (mg/mL)	Amount
Propidium Iodide (PI), 5 mg/mL(stock solution)	20 µg/mL	200 µL
Autoclaved Milli-Q water	n/a	50 mL
Total		50 mL

Propidium Iodide was dissolved thoroughly in sterile Milli-Q water to prepare a 5 mg/mL stock solution. A 20 µg/mL working solution was then prepared from the stock using autoclaved Milli-Q water as a diluent. The solution was filter-sterilized using syringe-driven filters within the Laminar Air Flow (LAF) cabinet and aliquoted into amber tubes. Since Propidium Iodide (PI) is light-sensitive, precautions were taken to avoid exposing it to light.

Additionally, considering that PI is a potential carcinogen, protective gloves were worn to prevent skin contact during handling.

Table 2.11: Half strength Murashige & Skoog (MS) medium

Reagent	Final concentration	Amount
Murashige & Skoog Basal Salts Medium	0.5x	2.15 g
Sucrose	1 % (weight/volume)	10 g
Agar Plant cell culture tested, powder	0.7 % (weight/volume)	7 g
Milli-Q water	n/a	1000 mL
Ampicillin	100 µg/mL	1000 µL
Total	n/a	1000 mL

The first four components were dissolved thoroughly in 900 mL of Milli-Q water in a reagent bottle, and the pH was adjusted to 5.7 before the volume was made up to 1000 mL. After adjusting the pH, Agar was added. The media was then autoclaved and allowed to cool. Once the reagent bottle was lukewarm to the touch, 1000 µL of filter-sterilized Ampicillin was added, and the solution was gently mixed. The media was poured into 50 mL portions in 20 sterile petri-dishes and allowed to solidify. After it cooled, the petri-dishes were wrapped with PhytaWrap.

Table 2.12: Callus induction medium (CIM)

Reagent	Final concentration	Amount
Gamborg's B5 basal salt mixture	0.32 %(weight/volume)	3.2 g
D-Glucose	2 % (weight/volume)	20 g
MES hydrate	0.05 %(weight/volume)	0.5 g
1000x Gamborg's vitamin solution	1x	1 mL
Agar Plant cell culture tested, powder	0.7% (weight/volume)	7 g
Milli-Q water	n/a	1000 ml
Ampicillin	100 µg/mL	1000 µL
2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5 µg/mL	500 µL
Kinetin	0.05 µg/mL	50 µL
Total	n/a	1000 mL

The first four components were dissolved thoroughly in 900 mL of Milli-Q water in a reagent bottle, and the pH was adjusted to 5.8 with 1N KOH before the volume was made up to 1000 mL. After adjusting the pH, Agar was added. The media was then autoclaved and allowed to cool. Once the reagent bottle was lukewarm to the touch, 1000 µL of filter-sterilized Ampicillin, 500 µL of 2,4-Dichlorophenoxyacetic acid, and 50 µL of Kinetin were added. The solution was gently mixed, and 50 mL of the media was poured into 20 sterile petri-dishes, allowing it to solidify. After it cooled, the petri-dishes were wrapped with PhytaWrap.

Table 2.13: Shoot induction medium (SIM)

Reagent	Final concentration (in %)	Amount
Murashige & Skoog Basal Salts Medium	1x	4.3 g
Sucrose	1 % (weight/volume)	10 g
MES hydrate	0.05 % (weight/volume)	0.5 g
1000x Gamborg's vitamin solution	1x	1 mL
Agar Plant cell culture tested, powder	0.7% (weight/volume)	7 g
Milli-Q water	n/a	1000 mL
Ampicillin	100 µg/mL	1000 µL
d-Biotin	1 µg/mL	100 µL
Trans-zeatin	2 µg/mL	2000 µL
Total	n/a	1000 mL

The first four components were dissolved thoroughly in 900 mL of Milli-Q water in a reagent bottle, and the pH was adjusted to 5.8 before the volume was made up to 1000 mL. After adjusting the pH, Agar was added. The media was autoclaved and allowed to cool. Once the reagent bottle was lukewarm to the touch, 1000 µL of filter-sterilized Ampicillin, 100 µL of d-Biotin, and 2000 µL of Trans-zeatin were added. The solution was gently mixed,

and 50 mL of the media was poured into 20 sterile petri-dishes, allowing it to solidify. After it cooled, the petri-dishes were wrapped with PhytaWrap.

Additionally, 3 mL of the SIM media was poured into 35 mm round petri-dishes and allowed to solidify. After the media cooled, the petri-dishes were wrapped with PhytaWrap. These dishes were later used during imaging.

II. 3.2. Microscope for time lapse imaging and ablation

To fully resolve the emission spectra and localization of different fluorescent-tagged proteins in the callus, the microscope imaging system should be upright and equipped with: 1) a confocal module; 2) suitable excitation lasers; 3) spectral detectors; 4) a motorized dichroic mirror turret; 5) a motorized stage for multi-position image acquisition; 6) a definite focus nosepiece for fast auto-focus to compensate for focus drift during imaging; 7) a scientific camera; 8) a water-dipping objective (Preferably 40X magnification) 9) a universal mounting frame, and 10) a programmable microscope control software. We used a Zeiss Laser Scanning Microscope (LSM) 880 upright confocal module, which uses a pinhole-plane image detection assembly based on a 32-channel high-sensitivity gallium-arsenide phosphide (GaAsP) spectral detector controlled by the Zen software (ZEN23). This protocol uses four lasers: 405 nm for ablation, 488 nm for GFP, 514 nm for YFP, and 561 nm for the PI stain.

Table 2.14: Excitation and emission spectra combinations for confocal-based imaging

Channel	Excitation laser(nm)	Main Beam Splitter	Emission spectra for fluorescent protein/dye(nm)	Emission spectra for chlorophyll autofluorescence(nm)
DAPI	405	405	409-465	
GFP	488	488/561	490-510	650-750
YFP	514	458/514/594	519-540	650-750
PI stain	561	488/561	580-610	

II.4. METHOD DETAILS

II.4.1 GROWTH CONDITIONS

Seeds of *Arabidopsis thaliana* (Columbia ecotype) were used in all experiments. Following surface sterilization as described in Section II.2.2.1, the seeds were placed on half-strength Murashige and Skoog (MS) medium, adjusted to pH 5.7 and solidified with 0.8% agar. The plants were grown in a Percival AR-100L3 growth chamber under controlled conditions at 22°C with 70% relative humidity, exposed to continuous white light at an intensity of 45 $\mu\text{mol}/\text{m}^2/\text{s}$.

II.4.2 CULTURING OF SAMPLES

All procedures were conducted aseptically within a Laminar Air Flow (LAF) cabinet, except during sample incubation. The entire culturing process took approximately 28-30 days.

II.4.2.1 Seed Sterilization

For seed sterilization, I aliquoted approximately 300 seeds of *Arabidopsis thaliana* into a 1.5 mL microcentrifuge tube. The seeds were treated with 1 mL of 70% ethanol, mixed by inverting the tube for 2-3 minutes, and centrifuged at $4,226 \times g$. The ethanol was discarded, and the seeds were then treated with 1 mL of 0.8% sodium hypochlorite, vortexed for 2-3 minutes, and briefly centrifuged before discarding the sodium hypochlorite. The seeds were washed 5-7 times with 1 mL of sterile autoclaved Milli-Q water, with brief centrifugation at $4,226 \times g$ after each wash to ensure complete removal of sterilizing agents. The seeds were then cold-treated by storing them in 1 mL of sterile autoclaved Milli-Q water at 4°C for 2-4 days in the dark to promote synchronous germination, avoiding cold treatment after plating to prevent delayed germination.

II.4.2.2 Seed Plating and 1/2 MS Incubation

After cold treatment, approximately 30 sterilized seeds were transferred onto solidified half-strength Murashige & Skoog (MS) medium containing 0.7% agar using a 1 mL pipette. The seeds were spaced in two rows of 15, with a 0.5 cm gap between each seed. The plates were sealed tightly with PhytaWrap to prevent contamination. The plates were incubated vertically in a growth chamber at 22°C with 70% relative humidity, under continuous white light at $45 \mu\text{mol}/\text{m}^2/\text{s}$ for 8 days, or until 7 days post-germination (dpg). At 7 dpg, the

seedlings exhibited visible lateral root outgrowth. Care was taken to ensure the seedlings remained free from stress or contamination for optimal shoot regeneration. Water accumulation was periodically removed aseptically within the LAF to prevent bacterial contamination.

II.4.2.3 Callus Induction Medium (CIM) Incubation

At 7 dpg, the seedlings were transferred into sterile square petri dishes (120 mm × 120 mm) containing Callus Induction Medium (CIM) inside the LAF. Using heat-sterilized forceps, the seedlings were gently picked by their leaves and arranged in a linear pattern, placing 10-12 seedlings per plate. The seedlings were pressed gently to ensure root contact with the CIM surface. The shoots and hypocotyls were excised at the root-shoot junction using sterilized Vanna's scissors and discarded. The plates were sealed with PhytaWrap and incubated vertically in a growth chamber under the same growth conditions for 7 days, maintaining consistent humidity and light conditions.

II.4.2.4 Shoot Induction Medium (SIM) Incubation

After 7 days on CIM, the plates were opened inside the LAF, any accumulated water was discarded, and individual explants were transferred to new sterile petri dishes (120 mm × 120 mm) containing Shoot Induction Medium (SIM). The explants were arranged in a linear pattern with 10-12 explants per plate. The plates were sealed with PhytaWrap and incubated vertically for 8 days in the growth chamber under the same light and temperature conditions. By 7-8 days post-transfer to SIM, shoot progenitors began to appear, marking the successful initiation of shoot regeneration.

II.4.3. SHOOT REGENERATION ASSAY

The regeneration assay was performed following the method described by Kareem et al. (2015). Root and hypocotyl explants were harvested from 7-day-old seedlings (7 days post-germination) grown on half-strength Murashige and Skoog (1/2 MS) medium, composed of 2.15 g MS salts, 10 g sucrose, and 0.7% plant-based agar. These explants were then cultured on callus induction medium (CIM), which contained 3.2 g Gamborg B5 salt mixture, 20 g D-glucose, 0.5 g MES hydrate, 1 ml of 1000x Gamborg's vitamin solution, and 0.7% plant-based agar, supplemented with 0.5 µg/ml 2,4-D and 0.05 µg/ml kinetin. After 8 days on CIM, explants were transferred to shoot induction medium (SIM) and incubated for 30 days. SIM consisted of 4.3 g MS salts, 10 g sucrose, 0.5 g MES hydrate, 1 ml of 1000x Gamborg's vitamin solution, 1 µg/ml d-Biotin, and 0.7% plant-based agar, with 10 µM trans-zeatin serving as an external hormonal stimulus. For gene overexpression or silencing driven by the G1090:XVE artificial promoter, SIM was supplemented with 5 µM estradiol for either 24 or 48 hours on the 8th day of SIM incubation or continuously throughout the 30-day period. The number of shoots per 2 cm explant was quantified after 30 days on SIM and presented graphically in the figures.

II.4.4. QUANTIFICATION OF GROWTH RATE

II.4.4.1. MorphoGraphX based quantification of growth rate

Volumetric data for progenitor and non-progenitor cells during de novo shoot formation were obtained and visualized using MorphoGraphX 2.0 r1-254 (MGX) (Barbier de Reuille

et al., 2015; Strauss *et al.*, 2022). Time-course confocal stacks of transgenic material and cells stained with PI (20 µg/ml) to highlight cell boundaries were enhanced 2-4 times and blurred with a Gaussian filter (0.5 µm radius). The resulting blurred stacks were segmented using the ITK watershed auto-seeded algorithm, with threshold levels ranging from 800 to 2000, followed by manual curation. Volumetric cell surface meshes were generated using the Marching Cubes 3D algorithm, with a cube size of 1 µm. In two-time course experiments, the PIN1-GFP signal was employed instead of PI for segmentation of progenitor cells, as PI weakly labeled these cells. Cell volumes were extracted via Cell Analysis 3D/Volume and visualized as heat maps or exported for growth rate calculations. Manual tracing of segmented meshes allowed for tracking individual cell lineages to measure growth and proliferation. Growth was calculated using the formula $(V_{tf} - V_{ti})/V_{ti}$, where V_{tf} is the final volume and V_{ti} is the initial volume. Growth rates per hour were determined using $[(V_{tf}/V_{ti})^{(1/n)}] - 1$, with n representing the number of time intervals (24 for hourly rates). Three time-course experiments, each with four time points (0 h, 5 h 30 min, 9 h, and 24 h), were analyzed.

II.4.4.2. Manual quantification of growth rate

Manual surface area measurements with ZEN2.3 SP1 black edition image processing software. Quantification was performed on Z-stack maximum intensity projection images of callus tissue, where progenitor cells were stained with 20 µg/ml filter-sterilized Propidium Iodide (PI) at 0 h, 5 h 30 min, 9 h, 24 h, and 48 h. Only surface cells were analyzed. For surface area measurements, the closed Bezier tool in ZEN2.3 SP1 was used to outline cell boundaries, enabling calculation of the top surface area. The percentage increase in surface

area for individual cells and the entire progenitor group over time was presented graphically. Cell length and width were measured using the line tool in ZEN2.3 SP1 by drawing edge-to-edge lines across the cell center both lengthwise and widthwise. Measurements of progenitor cells focused on the 5–8-hour window when the highest number of progenitor cells was observed before division, as longer intervals were excluded due to rapid cell division.

II.4.5. QUANTIFICATION OF MICROTUBULE ANISOTROPY

Cortical microtubule organization was quantified using the FibrilTool macro plug-in in Fiji/ImageJ, following the method described by Boudaoud et al. (2014). For each cell, the region of interest was defined using the Polygon selection tool in Fiji/ImageJ. The degree of anisotropy and orientation angle were measured in PIN1-GFP-labeled progenitor cells and compared to surrounding non-progenitor cells lacking PIN1-GFP expression. Anisotropy values ranged from 0 (completely isotropic) to 1 (fully anisotropic), allowing for direct comparison between the two cell types.

II.4.6 TIME LAPSE IMAGING OF PROGENITORS

The procedure for time-lapse imaging of progenitors began by preparing callus samples that had been incubated on SIM medium for 5-7 days. I excised a 0.25-0.5 cm section of green tissue from the callus, under sterile conditions, and placed it in the center of a sterile petri dish containing 3 mL of solid SIM medium. The sample was then fixed in place by cooling

a drop of molten agar to approximately 37°C and carefully sealing the tissue without fully encapsulating it, ensuring the top surface remained clear for imaging.

Time-lapse and time-point images were obtained using the Zeiss LSM 880 and Leica TCS SP5 II confocal laser-scanning microscopes, following the protocols described by in (Mathew *et al.*, 2023). For live imaging of the callus, a 40x water-dipping objective was used, while root time-point images were captured with a 20x (air) objective. Progenitor cells were located using a 10x air objective, and subsequent images were captured using a 40x water-dipping objective. A small piece of green callus was excised from the explant and placed in a sterile 35mm petri dish containing SIM or SIM supplemented with steroids. Slightly cooled, melted agar droplets were applied to fix the sides of the callus onto the SIM medium, preventing the sample from floating during live imaging with the 40x objective. The progenitor cells were tracked as they matured into the meristem, and each stage of progenitor development was captured using the same settings as for Day 1 of progenitor spotting. The progenitor stage was identified by the number of superficial progenitor cells.

Imaging was conducted with 50% laser power of the Argon laser (488nm for GFP, 514nm for YFP, and 561nm for PI), using a master gain of 600-800, a pinhole range of 50-200, and a frame size of 1024x1024 pixels. Line averaging was set to 2, and digital zoom was adjusted to 0.6. The master gain and pinhole settings were optimized based on the sample. Z-stack images were taken at 5µm or 1µm intervals, and autofluorescence was captured at a 633nm wavelength.

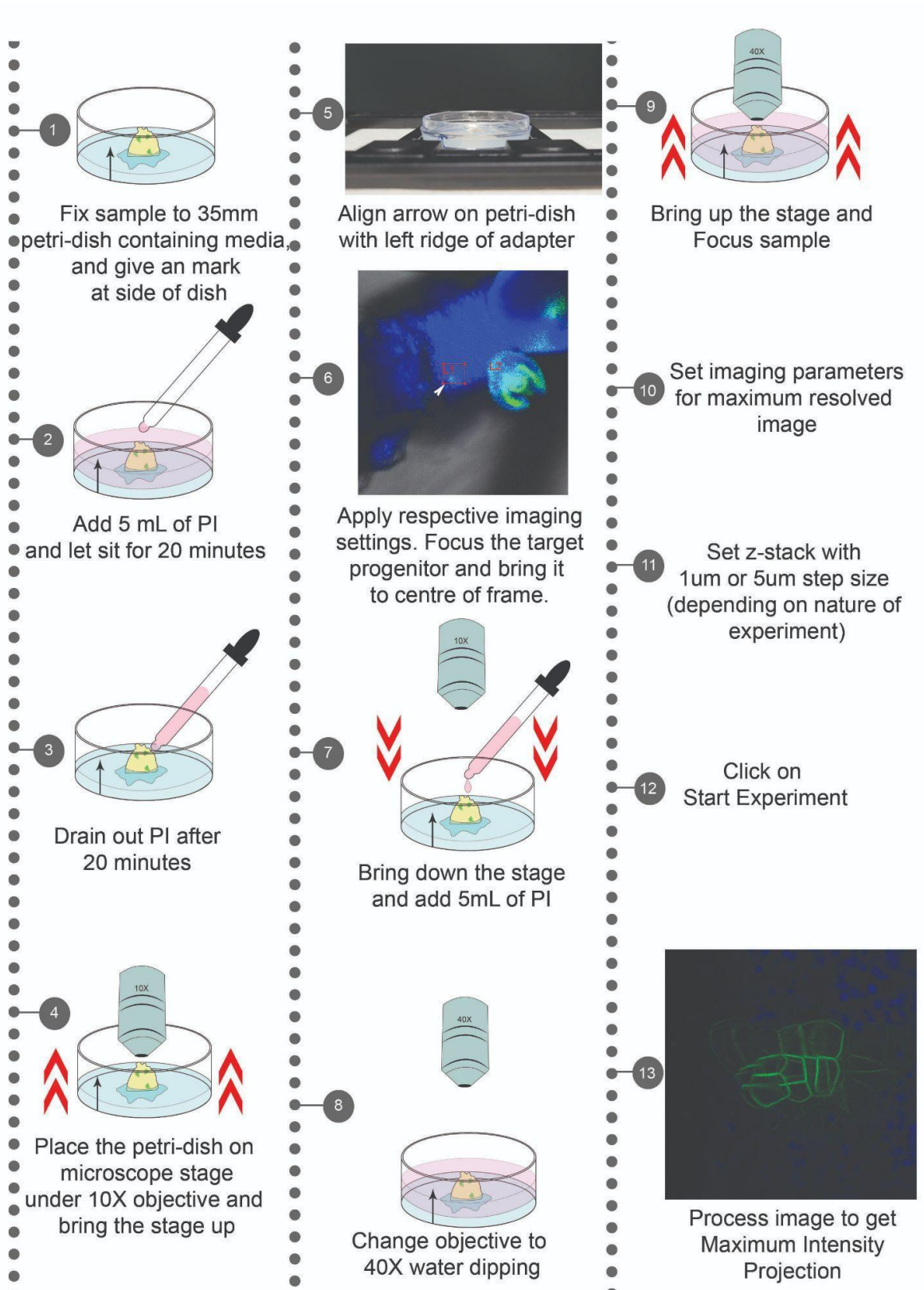


Figure. 2.1: The schematic demonstrating the entire procedure for imaging of a progenitor.

Filter-sterilized 20µg/ml propidium iodide (PI) was used to stain the cell walls (Figure 2.1). The image data was saved in .czi format for further analysis and 3D reconstruction. After imaging, the sample was washed with autoclaved Milli-Q water and sealed with PhytaWrap to prevent contamination, ensuring the sample remained in place for continued incubation in the growth chamber.

II.4.7. QUANTIFICATION OF PIN1 LOCALIZATION PATTERN AND INTENSITY

To quantify PIN1-GFP localization and polarity, a two-step approach was employed using Fiji/ImageJ software (Figure 2.2, Figure 2.3, Figure 2.4). First, a z-stack image of the progenitor was acquired with a 1 µm step size in either .czi or .lsm format and processed using Zen 2.3 SP1 Black Edition. The boundaries of the cells were clearly marked using propidium iodide (PI) to delineate the plasma membrane. The image was processed by selecting individual cells, ensuring no interference from overlaying or underlying cells. The "Subset" tool in Zen was used to extract data for one cell at a time, ensuring clarity in the analysis. For spatial analysis, the saved snapshot of the marked cell was opened in Fiji/ImageJ. The "Angle Tool" was used to measure the angles between the centroid and the measurement points in a clockwise direction. The angle data, along with the Mean Gray Value data, was recorded in an Excel sheet. The localization pattern was calculated by

plotting the Mean Gray Value of PIN1-GFP against the 0°-360° angle. This graph allowed identification of the side of the cell with the highest intensity, which corresponded to the side with the strongest PIN1-GFP polarity.

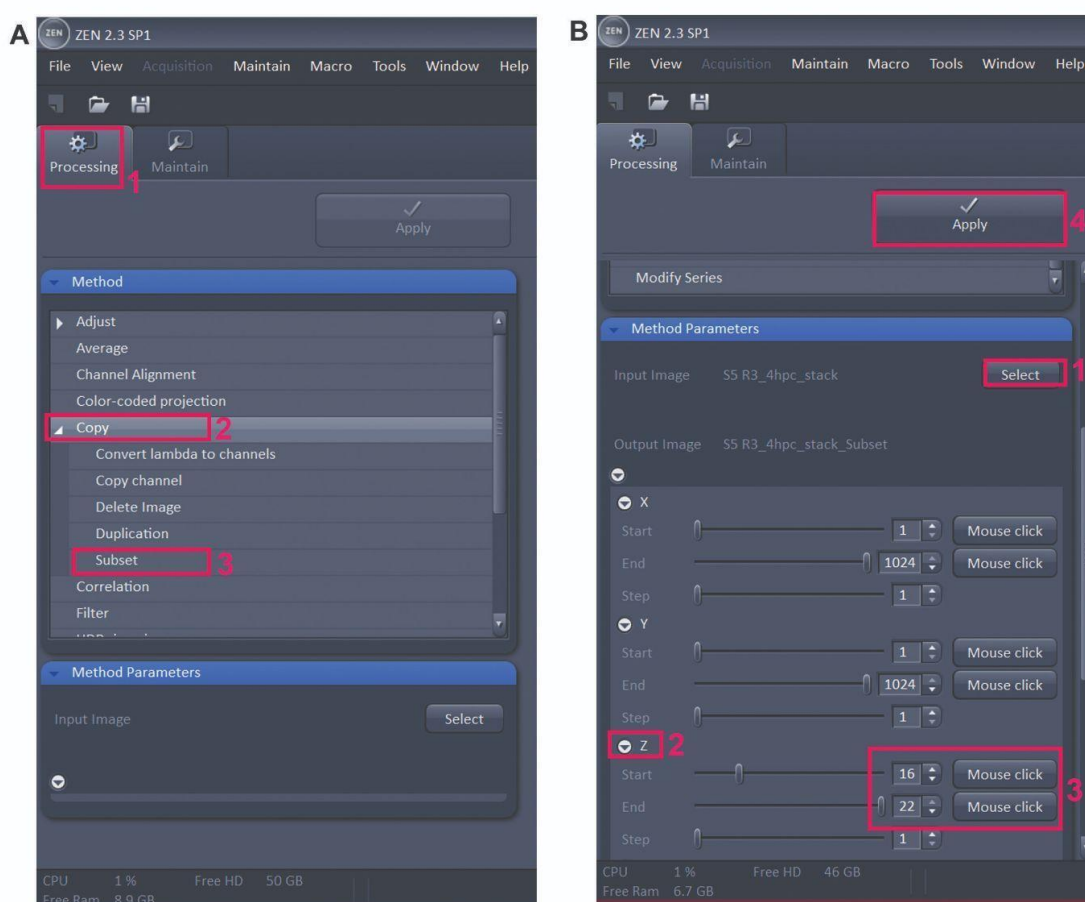


Figure. 2.2: Extracting a subset of z-positions from a complete set of Z-stack. (A, B) The screenshot detailing the steps to be followed to extract the desired set of z-positions from a complete z-stack. The numbers denote the order of commands to be followed.

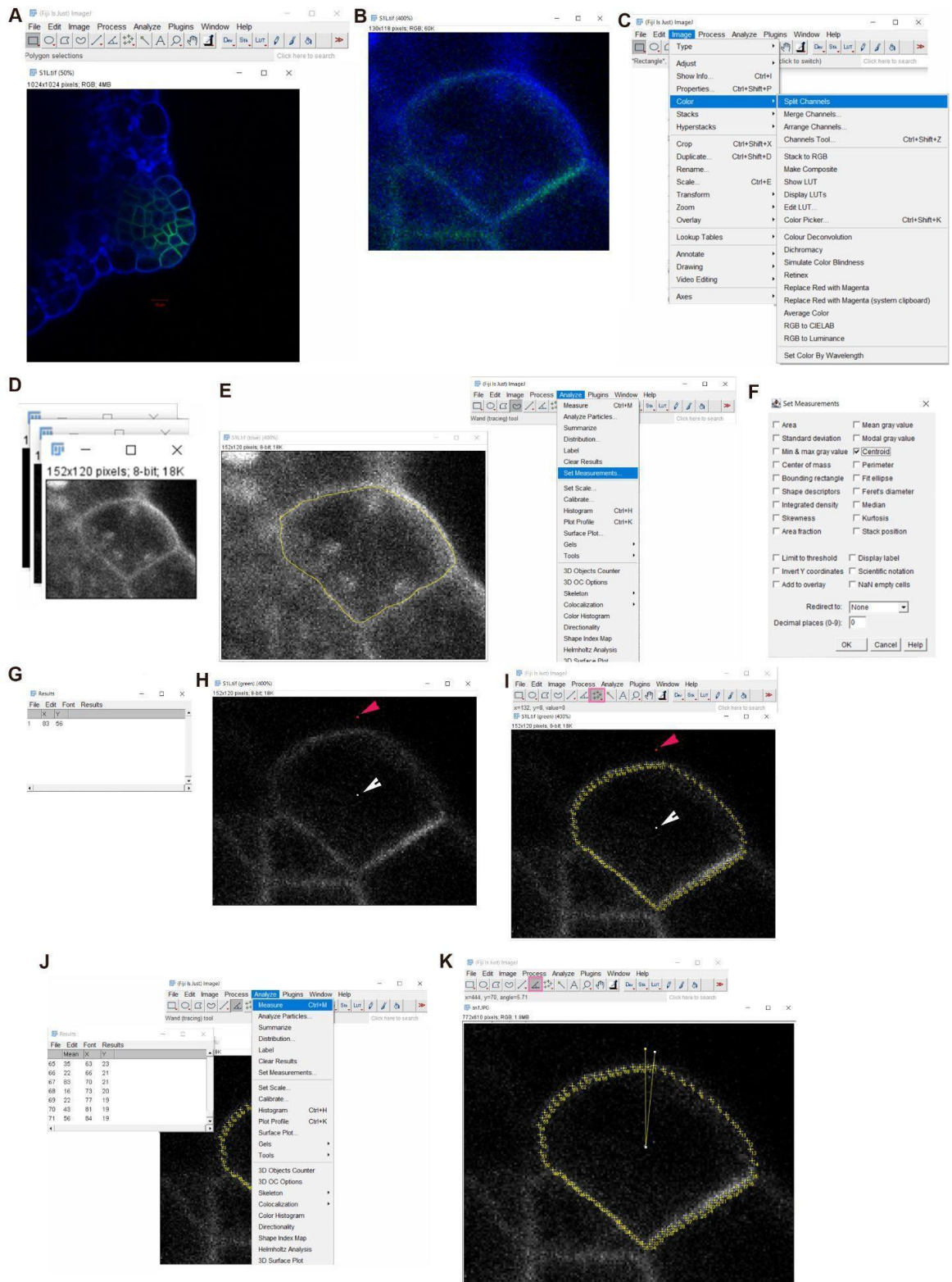


Figure. 2.3: Steps for PIN1-GFP intensity calculation using ImageJ. (A) Snapshot of the progenitor image opened using Fiji/ImageJ. (B) Zoomed in image of the cell to be quantified. (C-D) Steps to split channel (C), screenshot to split channels into Red, Green and Blue (D). (E-G) Calculation of centroid of the cell. (H) Coordinates of centroid marked on the cell using white dot (indicated by a white arrowhead). A red dot (indicated by a red arrowhead) is marked exactly above the centroid, outside the cell for further analysis. (I) Different points of membrane were marked using multipoint tool (in red border). (J) Steps for measuring the intensity of the multipoints marked on cell membrane. (K) Snapshot for measurement of angle for each point using angle tool (in red border).

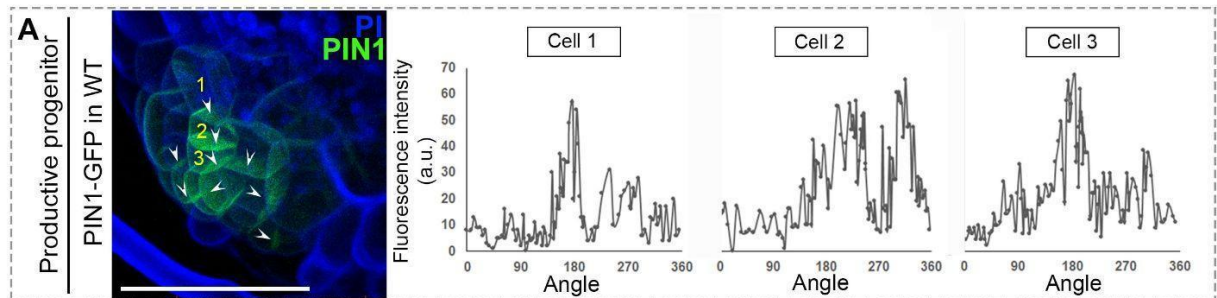


Figure. 2.4: Direction of PIN1-GFP polar localization in a cell. (A) A confocal micrograph showing a PIN1-GFP (green) marked progenitor. The white arrowheads denote the direction of PIN1-GFP polar localization. The blue color to the cell boundary is due to PI staining, and the blue puncta denotes the chlorophyll autofluorescence. (B) The graphs indicating the PIN1-GFP signal intensity if each of the measurement points against its angle. The angle denotes the position of each measurement point on the plasma membrane. The cell 1, cell, 2 and cell3 correspond to the cells 1, 2, and 3 in (A). Scale bar: 50 μ m.

To confirm the results, the quantification process was repeated for other progenitor cells. Analyzing multiple cells provided a comprehensive understanding of the PIN1-GFP localization and its polar distribution along the plasma membrane. This method provided both qualitative and quantitative insights into the distribution of PIN1-GFP, helping to map its polarity across progenitor cells. The tools used in Fiji/ImageJ, including the "Multipoint Tool," "Angle Tool," and "Set Measurements," were essential in deriving accurate measurements for this analysis.

II.4.8. TARGETED CELL ABLATION

For ablating the callus cells, the UV laser ablation procedure was carried out following the method described by Volpe et al. (2020). Laser ablation was performed using a Zeiss LSM 880 confocal laser-scanning microscope. Callus tissue was fixed in a 35mm petri dish containing SIM, as outlined earlier. To locate the progenitors, a 10X air objective was used, and prior to the ablation, an image was captured with a 40X water-dipping objective. The imaging parameters included 50% laser power, 488nm excitation, a master gain of 700, a pinhole corresponding to 1 AU, a frame size of 1024x1024 pixels, two averaging cycles, and a digital zoom of 0.6 (Kareem et al., 2015). Laser ablation was conducted either at the central point of a PIN1-GFP-marked progenitor or within a cluster of 4-12 surrounding cells marked with XTH9-YFP. A 40X zoom was applied to focus a 405nm UV laser at 80% power onto the selected cells. The laser was applied in two 30-second pulses with a 30-second interval between each, using a 408-488nm emission range, master gain set to 800, and a 1AU pinhole. After the ablation, the same region was imaged again using the same settings to confirm the procedure (Figure 2.5).

For ablating the cells from the root, the following method was used. The samples were prepared on a glass slide, by staining with propidium iodide and covering with cover slip. Fully resolved cells were chosen for ablation using the FRAP module of the Leica TCS SP8 multiphoton microscope at wavelength 900nm and 80% laser power. Once the cell of interest is in focus in 63X oil magnification, an ROI was drawn in the middle of the cell using the ROI tool. Ablation was performed with the setting for bleaching, where the

iterations for the pre-bleach, bleach, and post bleach were 1, 50, and 2 respectively. The line average was kept at 1. If the cell is not ablated, the cycle was repeated after 30 seconds.

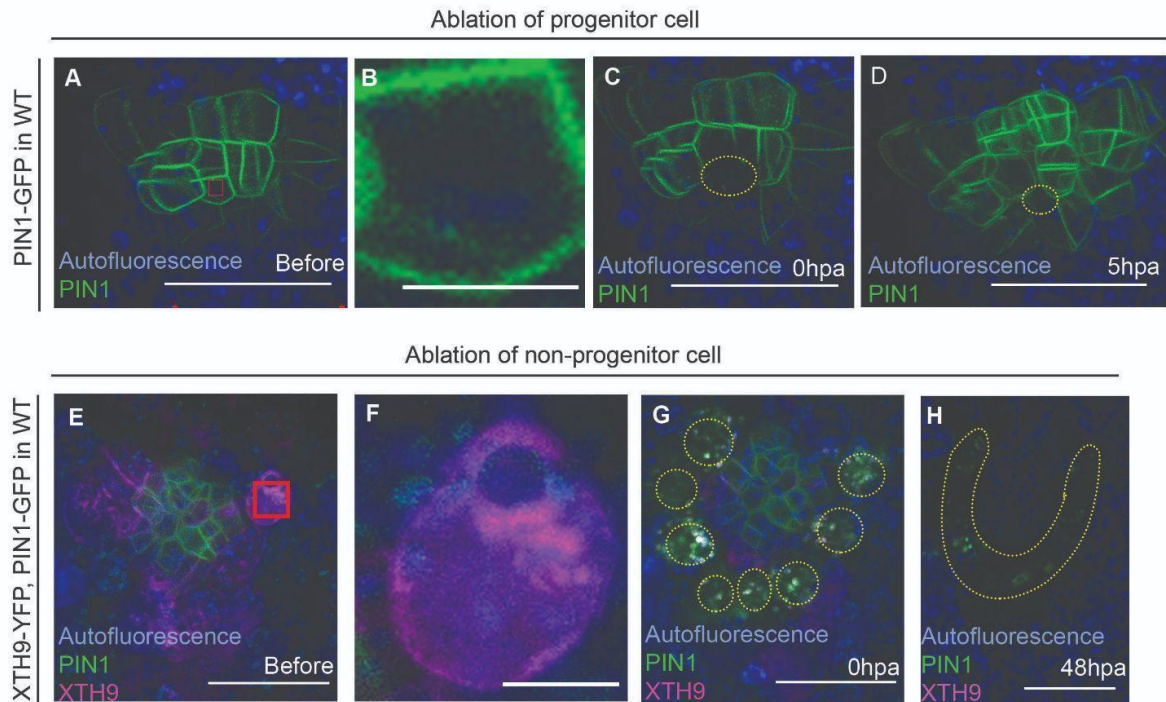


Figure. 2.5: Targeted laser ablation of progenitor and non-progenitor cells. (A-D) Ablation of PIN-GFP (green) marked progenitor cell. Z-stack image of the progenitor before ablation (A) Cell to be ablated zoomed in to fill the entire frame (B) Z-stack image of the progenitor 0 hours post ablation (0 hpa) (C) Z-stack image of the progenitor 5 hpa (D) **(E-H)** Ablation of XTH9-YFP (magenta) marked non-progenitor cell. Z-stack image of the progenitor before ablation (E) Cell to be ablated zoomed in to fill the entire frame (F) Z-stack image of the progenitor 0 hpa (G) Z-stack image of the progenitor 48 hpa (H) Red square indicates the cell to be ablated. Yellow dotted ellipse indicates the site of ablation. Fluorescent puncta at the site of ablation represent the autofluorescence emitted from cell debris. Scale bars: 50µm (A,C,D-E, G-H), 10µm (B,F).

II.4.9. FLUORESCENCE LIFETIME IMAGING MICROSCOPY (FLIM)- BASED CONFOCAL IMAGING

Samples were incubated with N⁺BDP or CarboTag-BDP, diluted to 10 μ M in liquid SIM, for 4–6 hours. Following incubation, samples were washed with either autoclaved Milli-Q water or liquid SIM before FLIM analysis. FLIM experiments were performed using a Leica SP8-DIVE multiphoton microscope. Excitation was carried out at 488 nm, and fluorescence emission was collected between 500 and 550 nm using a hybrid detector. Images (256 $\times$ 256 pixels) were captured over 60–120 seconds, depending on signal strength. The FLIM data were processed with SPCImage 8.5 software to generate two-component exponential decay curves for each pixel. Final FLIM images are presented in a false-color scale, reflecting the mean fluorescence lifetime per pixel in nanoseconds.

II.4.10. ROOT TIP RESECTION AND IMAGING

The seedlings were carefully placed on a sterile glass slide with a few drops of sterile water, and their root tips were manually resected using a sterile 1ml syringe (Dispovan) under a Zeiss Axio Scope.A1 microscope. The resection was performed within the root meristem, from the QC to the elongation zone. High and low cuts were defined by the distance from the fourth tier of columella cells. To determine the resection position, an image of the detached root tip segment was captured immediately after the cut. Following resection, the seedlings were either returned to MS medium or prepared for imaging. For confocal imaging, the resected seedlings were placed on a sterile glass slide, mounted with filter-

sterilized 20µg/ml propidium iodide (PI), and imaged using a Zeiss LSM 880 or Leica SP5 laser scanning microscope.

II.4.11. TREATMENTS

II.4.11.1. Steroid treatment of shoot progenitors during *de novo* shoot regeneration

The progenitors, identified through confocal-based live imaging, were treated with liquid SIM containing 5µM Estradiol by direct local application for 20 minutes (short pulse). After the treatment, the samples were washed with sterile milliQ water and transferred to solid SIM supplemented with 5µM Estradiol for prolonged induction or to solid SIM alone for continued incubation.

II.4.11.2. Hyperosmotic treatment of shoot progenitors during *de novo* shoot regeneration

For the hyperosmotic shock treatment of shoot progenitors, a solution of 0.4M mannitol in liquid SIM medium was prepared. The samples were incubated for 8 hours before imaging.

II.4.11.3. Steroid treatment of root in experiments for root tip restoration

For *35S::PLT2:GR* in WT, 2-day-old seedlings (for 48-hour Dexamethasone induction) or 4-day-old seedlings (for 2-hour Dexamethasone induction) were transferred to MS medium containing 20µM Dexamethasone and incubated for the required duration before resection.

II.4.11.4. Hormone treatment of root in experiments for root tip restoration

The 4-day-old seedlings, following resection, were transferred to ½ MS plates supplemented with 1µM or 5µM trans-zeatin. The seedlings were then incubated continuously from 0 hours post-culture (hpc) until 5 days post-culture (dpc).

II.4.12. RNA SEQUENCING

For the experiment, *35S::PLT2:GR* in WT (PLT2-oe) seedlings were treated with dexamethasone (DEX) for 2 hours prior to making high cuts on their root meristems. At 8 hours post-cut (hpc), approximately 2000–2500 root meristems were collected by excising them below the hypocotyl. Root meristems were also collected from *35S::PLT2:GR* in WT seedlings that had been induced for 48 hours. The 8hpc time point was chosen for collection, as PLT2 expression peaks at this stage (Durgaprasad *et al.*, 2019)

The quality of the raw FASTQ reads was assessed using FastQC v.0.11.9 with default settings. The raw reads were then preprocessed with Fastp v.0.20.1 (using parameters: `--trim_front1 8 --trim_front2 8 --length_required 50 --correction --trim_poly_g --qualified_quality_phred 30`), followed by re-assessment of quality with FastQC and summary generation through MultiQC.

The processed reads were aligned to the *Arabidopsis thaliana* (TAIR10; <https://www.arabidopsis.org/>) genome using the STAR aligner v.2.7.9a, with parameters: `--outSAMtype BAM SortedByCoordinate, --outSAMunmapped Within, --quantMode TranscriptomeSAM, --outSAMattributes Standard, --outFilterScoreMinOverLread 0.5`, and

`--outFilterMatchNminOverLread 0.5`. The rRNA and tRNA annotations were removed from the GTF file of *Arabidopsis thaliana* prior to alignment.

The resulting sorted BAM files from each sample were quantified using featureCounts v.2.0.1, with the filtered GTF file, to obtain gene counts. These counts were subsequently used in DESeq2 for differential expression analysis, applying a statistical significance threshold of $\alpha = 0.05$ and p-value adjustment using the Benjamini-Hochberg method. The 'regularized log' transformation from DESeq2 was employed for principal component and clustering analyses.

DESeq2 result files were filtered based on six combinations of p-value and fold change thresholds. For Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, the gene IDs from the DESeq2 results were submitted to bioDBnet for further analysis.

II.4.13. STATISTICAL ANALYSIS

Statistical analysis was conducted for all experiments and quantitative data. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Specific details, including sample size (n) and mean $\pm$ S.E.M., are provided in the respective figure legends. For the regeneration assay, Welch's Two Sample *t*-test, unpaired *t*-test, and Kruskal-Wallis test were applied. Growth rate analysis was performed using the Mann-Whitney *U* test. The length-to-breadth ratio was evaluated with a paired Two Sample *t*-test. Microviscosity quantification was analyzed using both the Mann-Whitney *U* test and the unpaired *t*-test.

Chapter III: Activation of cell polarity is essential for de novo shoot regeneration

This work is a part of the published manuscript:

Varapparambath, V.*, **Mathew, M.M.*†**, Shanmukhan, A.P.*, Radhakrishnan, D., Kareem, A., Verma, S., Ramalho, J.J., Manoj, B., Vellandath, A.R., Aiyaz, M., Radha, R.K., Landge, A.N., Mahonen, A.P., Heisler, M.G., Weijers, D., Prasad, K†. (2022).

Mechanical conflict caused by a cell-wall-loosening enzyme activates *de novo* shoot regeneration. **Developmental Cell**, 57(17), pp.2063-2080. doi:

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(* Equal contribution, †Co-corresponding author)

III.1 INTRODUCTION

"Life is the constant adjustment of internal relations to external conditions."
— Herbert Spencer

Totipotency, the ability of a cell to develop into a complete organism or differentiate into all lineages of an organism, is a defining feature of plant cells. This unique trait allows plant somatic cells to be cultured *in vitro*, enabling the regeneration of any of its organs, organ system, or even an entire plant. Various *in vitro* culture methods, such as somatic embryogenesis (Kadokura *et al.*, 2018; Radoeva *et al.*, 2020), trans-differentiation (Atta *et al.*, 2009; Kareem *et al.*, 2016; Rosspopoff *et al.*, 2017), and callus-mediated regeneration (Gordon *et al.*, 2007; Kareem *et al.*, 2015), have been developed to exploit this potential. Collectively referred to as tissue-culture-based regeneration, these methods provide experimentally amenable systems to study self-organization in multicellular contexts. Of these approaches, callus-mediated regeneration remains the classic text-book example of how plant cell totipotency can be harnessed to generate shoot and root systems via *de novo shoot organogenesis* and *de novo root organogenesis*, respectively. Advances in cell and molecular biology have further propelled this field, including the development of techniques that can trigger organogenesis even from single-cell protoplasts (Sakamoto *et al.*, 2022). *De novo organogenesis* thus forms the basis for clonal propagation, a technique widely employed in horticulture and crop genome editing, and its utility continues to expand.

I used *de novo* shoot regeneration from *Arabidopsis thaliana* root explants as a model to investigate the self-organization of cells into a functional organ system—specifically, the shoot meristem, which ultimately gives rise to the entire shoot system. *De novo* shoot regeneration is a sequential process which involves culturing explants *in vitro* while supplemented with the plant hormones auxin and cytokinin. During this process, shoots regenerate from an unorganized mass of cells called the callus, which is rendered pluripotent and regeneration-competent by the hormonal treatment. However, not all cells of the callus can give rise to shoot meristems. Only the middle layer of the callus can give rise to shoot meristems in response to shoot inductive cues (high cytokinin) (Zhai & Xu, 2021), with the sporadic expression of the shoot stem cell regulator WUSCHEL (WUS) marking regions of prospective shoot meristem formation (Gordon *et al.*, 2007; Zhang *et al.*, 2017; Lardon *et al.*, 2020). This patchy expression of WUS and other shoot promoting factors reflects the inherent heterogeneity of the callus. Within the callus, there are clusters of initial cells that self-organize into shoot meristems and, eventually, functional shoots. These clusters, the precursors of shoot meristems, are termed “shoot progenitors”.

How are specific callus cells selected to become shoot progenitors? and What regulates their progression to shoot meristems? My investigations surrounding these two questions revealed that progenitors tend to arise from the mitotically active regions of the heterogeneous callus. Interestingly, a callus produces a greater number of shoot progenitors than they achieve shoot meristems, suggesting that some progenitors do not complete the process and end up non-productive. Here, I reveal that an abundance of shoot stem cell

regulators like WUS does not distinguish productive from non-productive ones. Instead, it is the localization patterns of polarity proteins on the cell membrane that determine which progenitors will successfully progress into functional shoot meristems. The localization pattern of the polar proteins ensures productive fate to progenitors during the progression.

III.2. RESULTS

III.2.1. PIN1 localization pattern is essential for *de novo* shoot regeneration and distinguishes productive progenitor from pseudo-progenitors

In response to shoot inductive cues (high cytokinin), shoots regenerate from the middle layer of the callus (Zhai & Xu, 2021), specifically from a cluster of initial cells within the middle layer. This cluster of initial cells is termed shoot progenitor. These progenitors that arise within the unorganized callus are precursors to shoot meristems, which ultimately develop into functional shoots (Figure 3.1.A). The progression of shoot progenitor to shoot meristem is an example for self-organization in a multicellular setting. Although previous studies explored the expression of shoot specific genes during this scenario, the documentation was limited to shoot meristem stage and occasionally the stage just before shoot meristem- the promeristem (Gordon *et al.*, 2007; Kareem *et al.*, 2015), and for good reason. The regenerating callus has an irregular topology and uneven surface. Furthermore, my investigations revealed that the progenitors were not present on the callus surface but approximately 1-2 cell layers deep, making progenitor detection, tracking and live-imaging

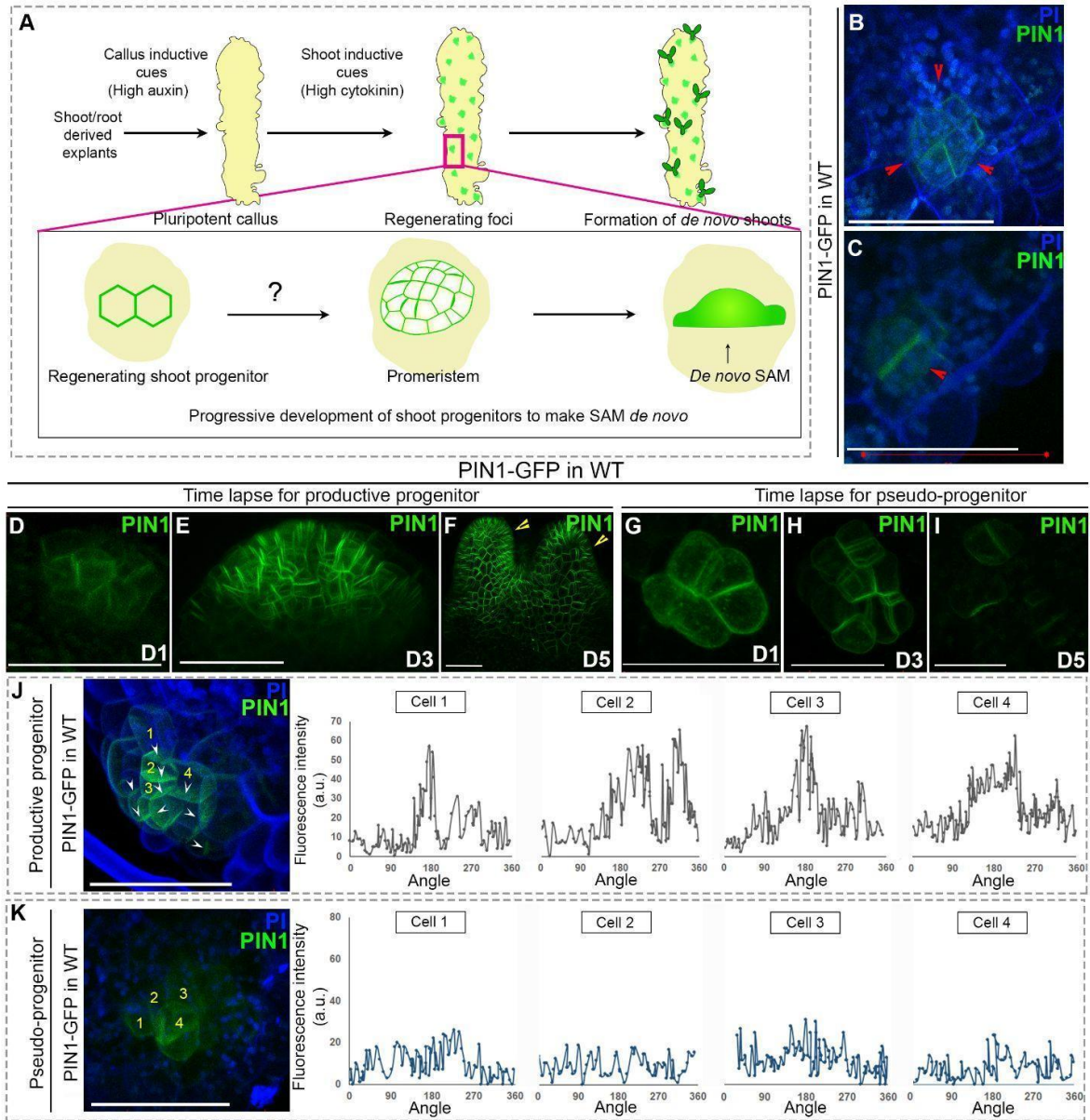


Figure. 3.1: Localization pattern of polarity proteins governs the shoot meristem formation (A) Schematic showing progression of PIN1 (green) marked progenitor to shoot meristem. (B-C) Representative images of 2-4 celled (B) and 8-10 celled (C) PIN1-GFP (red arrow heads) marked progenitor (n=34). (D-I) Representative time lapse images showing PIN1-GFP in productive progenitor (PP) and pseudo-progenitor (PSP). Yellow arrowheads: leaf primordia (F). (J-K) Representative images of PP and PSP with PI-stained cell walls. White arrowheads: Direction of PIN1-GFP polarization (left). Graph depicts the fluorescence intensity of PIN1-GFP along the membrane of the cells annotated (1, 2, 3, 4) (ns, $P=0.1163$, Kruskal-Wallis test PP-cells) ($x=94$ cells), (ns, $P=0.1764$, Kruskal-Wallis test PSP-cells) ($x=73$ cells) ($**P=0.000$, Kruskal-Wallis test between PP and PSP-cells). The

peak denotes the wall with maximum PIN1-GFP fluorescence (right). Scale bars: 50µm. n=sample number.

challenging (Figure 3.1.B- 3.3.C). Using high-resolution confocal microscopy, I successfully identified these buried progenitors marked with the green fluorescent reporter of the auxin efflux carrier PINFORMED1 (*pPIN1::gPIN1-GFP* in Wildtype). The PIN1-GFP translational reporter was chosen due to its established role in marking regenerating shoots and their predecessors (Gordon *et al.*, 2007; Kareem *et al.*, 2015).

While PIN1 shows expression throughout the callus during its formation in response to high auxin (Kareem *et al.*, 2015), it gets confined to specific initial cells in response to cytokinin marking the onset of progenitor formation (the 8th day of shoot induction medium, SIM). I observed that the first time PIN1-GFP localized to the plasma membrane is at the 2-4 cell stage of the progenitor, and rarely at the single-cell stage (Figure 3.1.B - 3.1.C). Through confocal based real time tracking of several progenitors, I found that only a sub-population of them progress into shoot meristem and ultimately functional shoots. Approximately one-quarter of the progenitors aborted their development midway, suggesting at least two distinct populations: (a) productive progenitors and (b) pseudo-progenitors (Figure 3.1D-3.1I). I documented the stage wise progression and the dynamics of these progenitors in space and time. The productive progenitors remain indistinguishable from the pseudo progenitors at 1-2 celled stages. However, by the 6-15 celled stage, a strong and sharp localization of PIN1-GFP to the plasma membrane of productive progenitors became evident. Quantitative analysis of PIN1-GFP across multiple progenitors revealed a consistent localization pattern, wherein PIN1 faced outward or sideways toward adjacent

cells, but never inward toward the progenitor (Figure 3.1.J). This pattern remained invariable across the productive progenitors tested although the number of cells with PIN1 facing outward or sideward varied. Notably, the productive progenitors at this stage had small, polygonal, compactly arranged PIN1-GFP marked cells. Over three days, the productive progenitors progressed into late progenitors (approximately 70 cells; Figure 3.1.E) and a promeristem (approximately 120 cells; Figure 3.1.F). The constituent cells continued to retain the shape and packing of the productive progenitors throughout its progression (Figure 3.1.D - 3.1.F). In contrast, the pseudo-progenitors of the 6-15 celled stage suffered from reduced PIN1-GFP expressions and its weak localization to the plasma membrane. No discernable PIN1 localization pattern was detected in the pseudo progenitors (Figure 3.1.K).

Moreover, unlike the productive progenitors, the constituent cells of the pseudo progenitors were large, bubble shaped and loosely packed (Figure 3.1.G) Thus, the 6-15 celled stage appears to be the earliest stage to distinguish productive from pseudo progenitors based on PIN1 expression and progenitor morphology. The pseudo-progenitors progress at most till the 20-cell stage and never beyond, with the already weak PIN1-GFP steadily vanishing. The complete disappearance of PIN1-GFP marks the abortion of the progenitor (Figure 3.1.G -3.1.I). Given the challenges in spotting and tracking the progenitors, I do not discount the possibility of other types of progenitors that do not fit into this binary classification.

Since the PIN1-GFP localization pattern demarcates productive progenitors from pseudo-progenitors, I asked whether deregulating the PIN1 localization pattern would impair shoot regeneration. Towards this, I perturbed the PIN1 localization via inducible

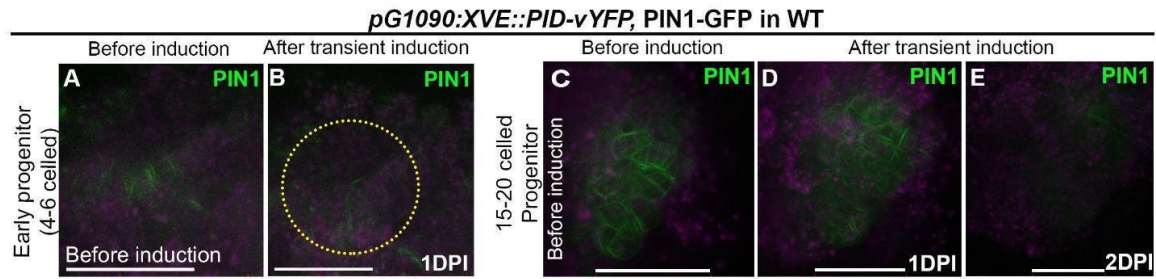


Figure. 3.2: Specific localization pattern of PIN1 correlates with *de novo* shoot meristem formation. (A-E) Representative time lapse image shows that transient overexpression of PID by local application of estradiol for 20 minutes on 4-6 celled progenitor (n=14)(A) or 15-celled progenitor(n=25)(C) abolished progenitor development. PIN1-GFP was found to be fading even after 1day post removal from steroid induction (B,D,E).Scale bars: 50μm. n=sample number.

overexpression of the PIN localization regulator, PINOID (PID). PID overexpression (*pG1090: XVE::PID-vYFP,pPIN1::gPIN1-GFP* in wild type), induced by estradiol, aborted progenitor development and prevented the formation of the shoot meristem (Figure 3.2.A - 3.2.F). A brief 20-minute pulse of PID overexpression in 4-6 cell stage or 15-cell stage progenitors already disrupted PIN1-GFP localization. This disruption in PIN1 localization became conspicuous 24 hours after removal from estradiol induction (Figure 3.2.B, 3.2.D). Corroborating these findings, loss-of-function mutants of *pin1* (*pin1-1* and *pin1,pin3,pin4,pin7*) or its polarity regulators *pid* (*pid,wag1,wag2*) was found to result in severe shoot regeneration defects(Varapparambath and Mathew et al., 2022). These results demonstrate the necessity of PIN1 and its self-organized localization pattern in ensuring progenitor progression to shoot meristem and complete shoot.

III.2.2. Cell polarity regulator SOSEKI promotes *de novo* shoot regeneration

In the previous section, I demonstrated the necessity of the localization pattern of PIN1 for successful shoot regeneration *de novo*. This became an incentive to check whether other polar localized proteins are involved in *de novo* shoot regeneration and if they can distinguish productive progenitors from pseudo ones. To explore this, I examined the role of SOSEKI (SOK) proteins in shoot regeneration from callus. SOK proteins are markers of intrinsic cell polarity and follow a polar localization pathway different from that of PIN1. Different SOK proteins have been shown to localize to different subcellular edges (Yoshida *et al.*, 2019). Among the SOK proteins tested, I found the expression of SOK5-YFP (*pSOK5:gSOK5-YFP*, *pPIN1::gPIN1-GFP* in Wildtype) to be associated with both productive and pseudo-progenitors (Figure 3.3.A - 3.3.F .iii). The difference was that, in the productive progenitor, SOK5 expression was most prominent in the PIN1-GFP (Figure 3.3.A). This pattern of expression continued throughout the progression of the progenitor (Figure 3.3.B). In contrast, in pseudo-progenitors, SOK5 expression was observed in the cells immediately surrounding the progenitor, and rarely in the PIN1-marked cells (Figure 3.3.C - 3.3.D). The SOK5 expression disappeared from these cells following the loss of PIN1 expression, which coincided with the progenitor's failure to progress (Mathew *et al.*, Unpublished data). In contrast, SOK2-YFP (*pSOK2::gSOK2-YFP*, *pPIN1::gPIN1-GFP* in Wildtype) exhibited a clear distinction between productive and pseudo-progenitors. Unlike SOK5, SOK2 was entirely absent from pseudo-progenitors and exclusively expressed in the

productive progenitors, specifically the PIN1-GFP marked cells of productive progenitors

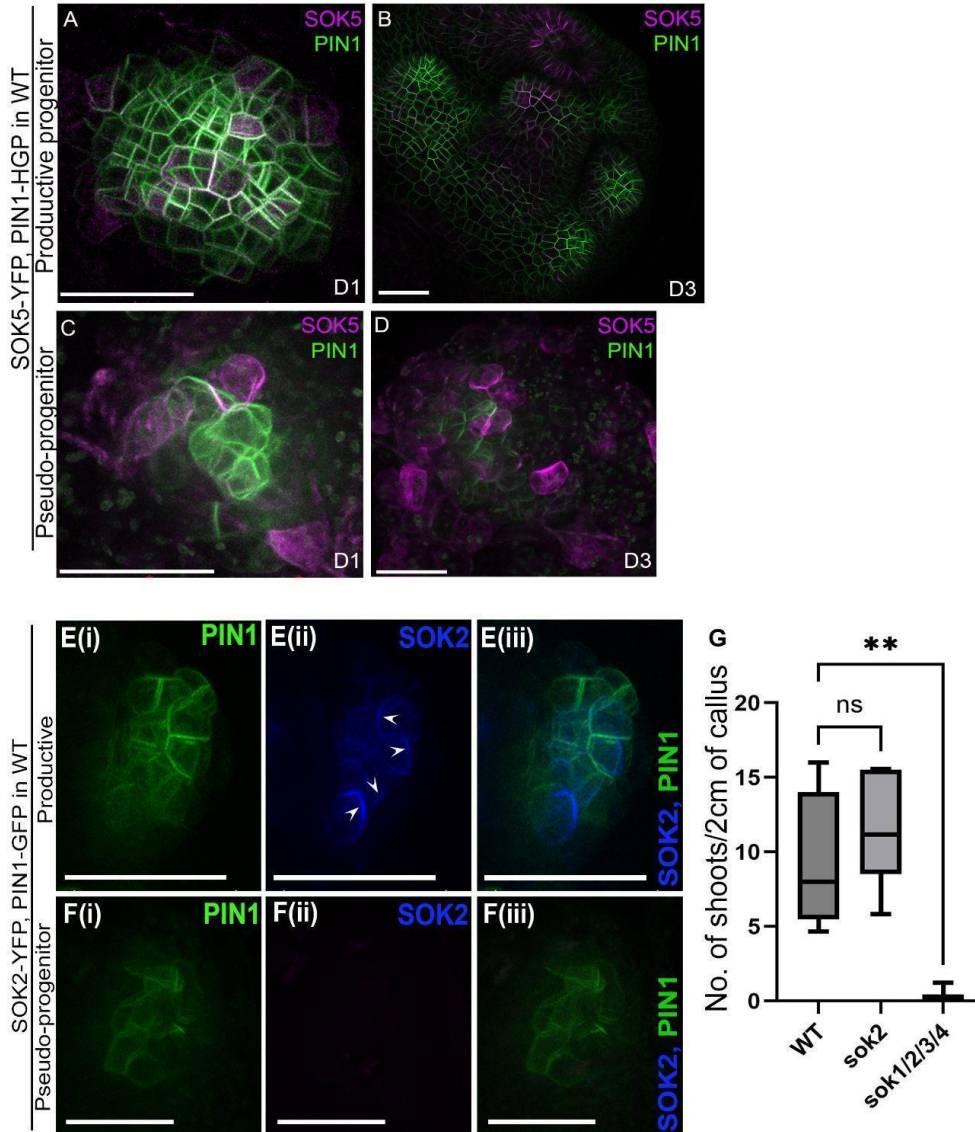


Figure. 3.3: Intrinsic cell polarity protein, SOSEKI can distinguish productive progenitors from pseudo-progenitors. (A-B) Representative images for SOK5-YFP in Productive progenitors at DAY1 (D1) of spotting the progenitor (A) and D3 of spotting the progenitor (B). (C-D) Representative images for SOK5-YFP in pseudo progenitors at D1(C) and D3 of spotting the progenitor (D). (E-F) Representative images for SOK2-YFP in Productive progenitors (n = 40) and pseudo-progenitors (n = 38). (G) Graphical representation of shoot regeneration in WT (n=120), sok2 single mutant (n=213) and sok1/2/3/4 quadruple mutant (n=194)(WT v/s sok2, ns p-value >0.9999; WT vs. sok1/2/3/4, **p-value=0.0065, Kruskal-Wallis test followed by Dunn's test). Scale bars: 50µm. n=sample number.

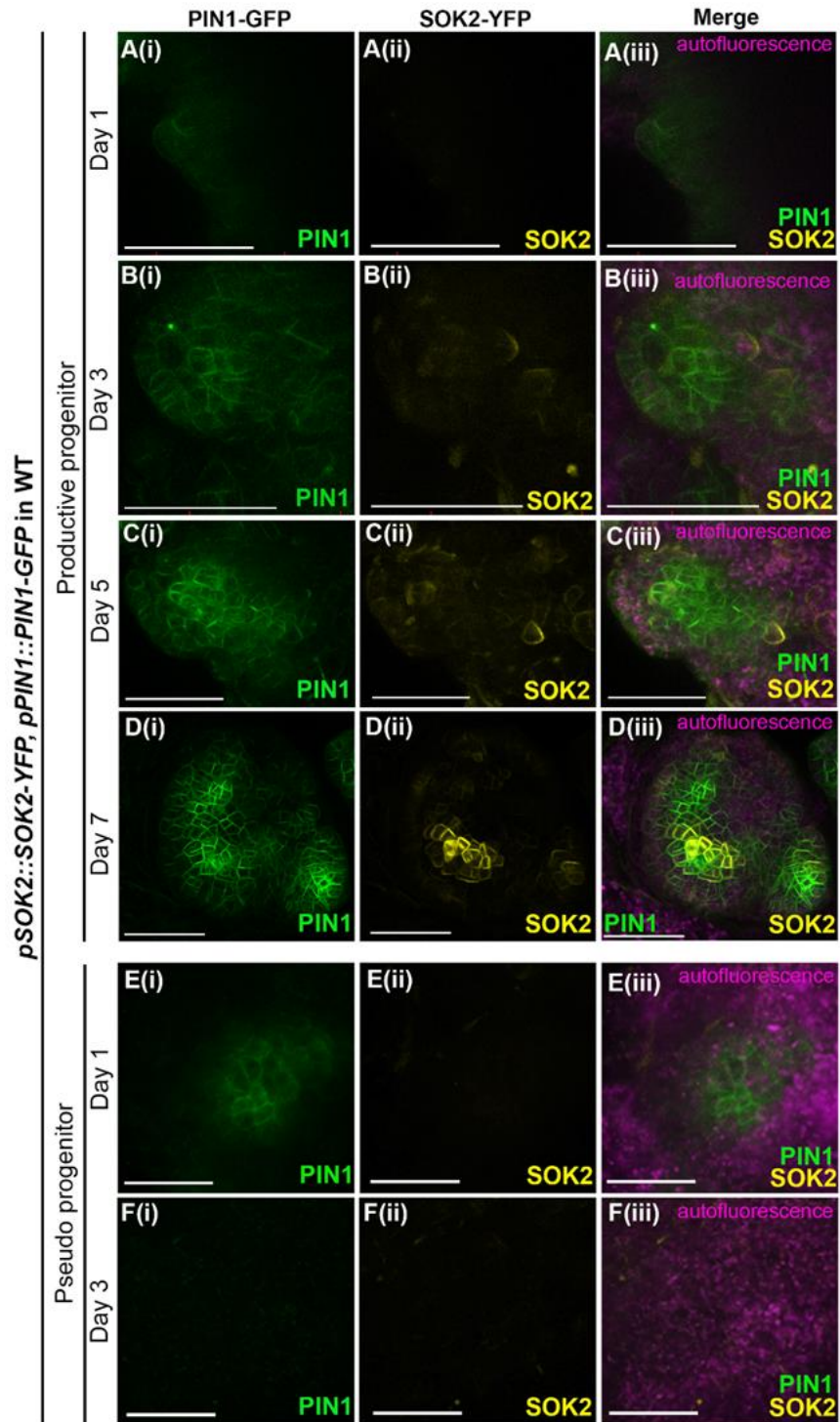


Figure. 3.4: The intrinsic cell polarity protein SOK2 is present only in the productive progenitors. (A-F) Representative time lapse of two sets of *pSOK2::SOK2-vYFP*, *pPIN1::PIN1-GFP* in WT showing that the PIN1-GFP (green) marked cells of productive

progenitors express SOK2-YFP (yellow) (n=40). SOK2-YFP was not expressed during early stages of progenitor (A(ii)-A(iii)). After 15-celled stage, few progenitor cells began to express SOK2 (B(ii)-B(iii)). The number of PIN1 marked cells expressing SOK2 increased over time, But SOK2 remained undetectable in majority of the PIN1 marked cells even in shoot (D(ii)-D(iii)). (E-F) Representative time lapse imaging shows that the pseudo-progenitor lacks SOK2 expression (n=38). GFP channel (A(i),B(i), C(i), D(i), E(i), F(i)), YFP channel (A(ii),B(ii), C(ii), D(ii), E(ii), F(ii)) merged image (A(iii),B(iii),C(iii),D(iii),E(iii),F(iii)). Magenta colour depicts auto-fluorescence. Scale bars:50µm, n=sample number.

(Figure 3.3.E - 3.3.F, 3.4.A - 3.4.F). SOK2 expression first appeared in productive progenitors at the 10-15 cell stage and was restricted to a subset of PIN1-GFP-marked cells, with no expression in other areas of the callus. This trend, where the number of PIN1-expressing cells outnumber the SOK2-positive cells, persisted throughout the progenitor progression (Figure 3.4.A - 3.4.D).

Given the specificity of SOK2 to productive progenitors, I investigated whether it plays a role in determining progenitor fate. A shoot regeneration assay showed that the *sok2* loss-of-function mutant did not impair shoot regeneration, likely due to functional redundancy among SOK genes (Figure 3.3.G). However, the *sok1/2/3/4* quadruple mutant exhibited a significant reduction in shoot regeneration compared to wildtype (Figure 3.3.G). These results suggest that SOK proteins, as regulators of intrinsic cell polarity, promote *de novo* shoot regeneration (Mathew et al., Unpublished data) Higher-order mutants that includes *sok5* are currently being generated in collaboration with Prof. Dolf Weijers at Wageningen University and Research, Netherlands (personal communication).

III.2.3. The progenitor cells are mitotically active

As mentioned previously, the callus is inherently heterogeneity and asynchronous in nature. The terrain of regenerating callus is irregular, with some regions exhibiting rapid growth and division, while others grow and divide more slowly. Additionally, the onset of progenitors

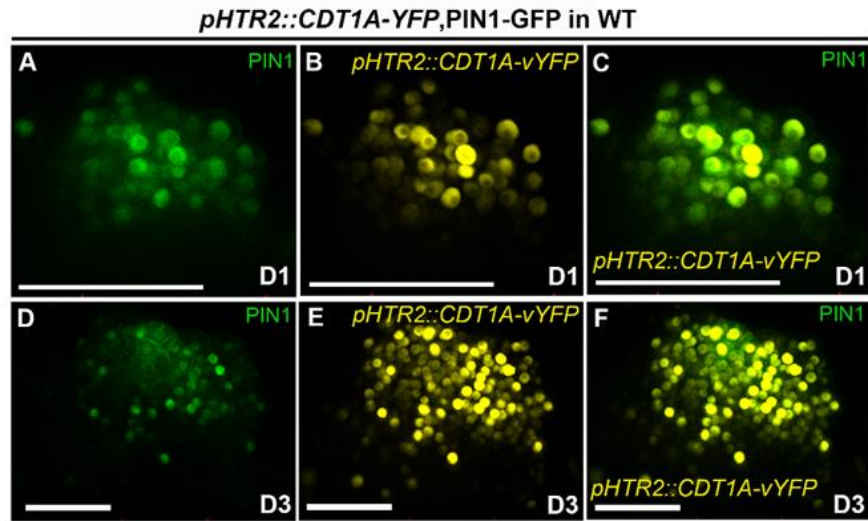


Figure. 3.5: Stochastic initiation of cell division at a point in the callus is essential to trigger the heterogeneity in terms of cellular behavior and molecular markers. (A-F) Time lapse imaging of *pHTR2::CDT1a-vYFP*, *PIN1-GFP* in WT(n=8). The expression of the cell cycle marker, CDT1a-YFP(yellow) , was observed even before PIN1-GFP (green) membrane localization at D1 of spotting (A). It should be noted that the green fluorescent puncta in (A) is the leakage of YFP into the GFP channel. (D-F) Membrane localization of PIN1-GFP marking progenitor initiation at D3 (D). GFP channel (A, D), YFP channel (B, E), Merged (C, F). Scale bars: 50µm. n=sample number.

occurs irregularly, with progenitors spaced apart in a seemingly random pattern. This prompted me to investigate whether there is any correlation between these two processes: Does the cell division status of regenerating callus relate to the positioning of progenitor inception? To explore this, I examined different regions of the regenerating callus using the S-phase cell cycle reporter, CDT1a (*pHTR2::gCDT1a-YFP*, *pPIN1::gPIN1-GFP* in wildtype) (Yin *et al.*, 2014) (Figure 3.5.A - 3.5.B). My observations revealed specific fields

of highly dividing cells scattered arbitrarily across the callus. Cells expressing CDT1a were concentrated in these $\approx 50\mu\text{m}$ diameter fields, compared to other areas of the callus (Figure 3.5.B - 3.5.C). Tracking these fields showed that the constituent cells acquire PIN1-GFP, which localizes to their plasma membranes, marking the onset of progenitor formation (Figure 3.5.D, 3.5.E). Since these microscopic fields indicate sites for progenitor inception, I refer to them as “morphogenic fields.” Notably, each morphogenic field produces only a single progenitor in its middle which can either be a productive progenitor or a pseudo-progenitor.

Next, I investigated whether the cell division status differed between productive and pseudo-progenitors. Utilizing CYCB1;1-YFP (*pCYCB1;1::CYCB1;1-vYFP*, *pPIN1::gPIN1-GFP* in wildtype), a G2/M-phase cell-cycle marker, I found that productive progenitors indeed differed from pseudo-progenitors in their cell division states (Figure 3.6.A - 3.6.G). A greater number of PIN1-GFP-marked cells in productive progenitors expressed CYCB1;1-YFP compared to those in pseudo-progenitors, with their numbers steadily increasing throughout the progression of productive progenitors. But the cells immediately surrounding the productive progenitors showed only sporadic CYCB1;1-YFP expression (Figure 3.6.A - 3.6.D). In contrast to the productive progenitors, the PIN1-GFP-marked cells in the pseudo-progenitors expressed CYCB1;1-YFP only during the 2-4 cell stage (Figure 3.6.F), and this expression ceased shortly thereafter in both the pseudo-progenitor and its surrounding cells. Ultimately, these pseudo-progenitors lost their PIN1 expression, indicating their failure to develop (Figure 3.6.G).

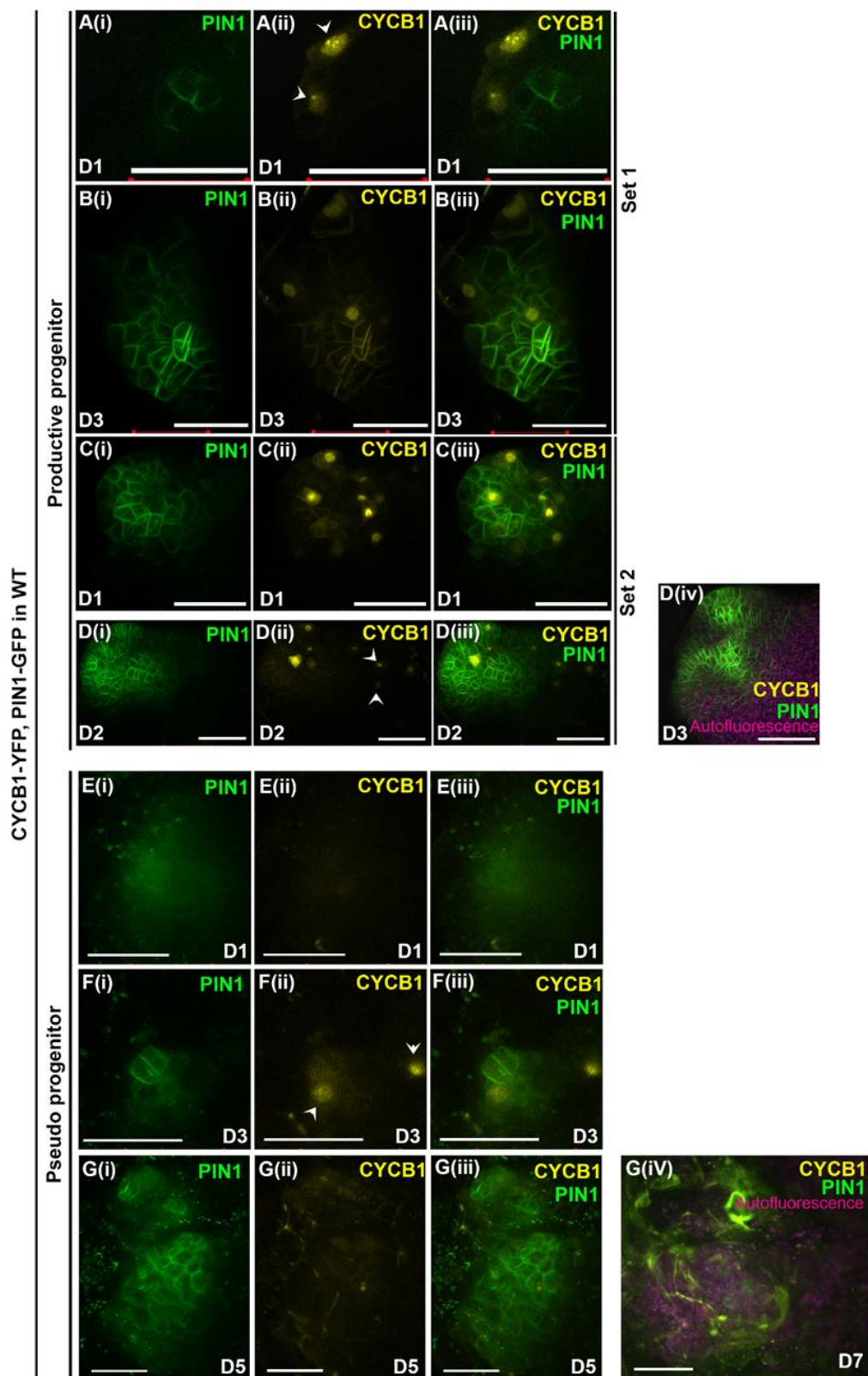


Figure. 3.6: Productive progenitor is actively dividing throughout all stages of its progression, while pseudo progenitors cease to divide and abort its progression. (A-D) Representative time lapse of two sets of *pCYCB1;1::CYCB1;1-vYFP*, *pPIN1::PIN1-GFP* in wildtype showing that the PIN1-GFP (green) marked cells of productive progenitors are actively dividing as indicated by CYCB1:1-YFP (yellow). While the PIN1-GFP marked progenitor cells undergo active cell division (B(i)-D(iii)), the surrounding non- progenitor cells sporadically divide as indicated by white arrow (A(ii),D(ii))(n=16). **(E-G)** Representative time lapse imaging shows that even though the pseudo-progenitor starts out dividing (F(i)-F(iii)), it ceases its division later (G(i)-G(iii)), thereby aborting its progression to shoot meristem (n=11)(G(iv)). GFP channel (A(i),B(i), C(i), D(i), E(i), F(i), G(i)), YFP channel (A(ii),B(ii), C(ii), D(ii), E(ii), F(ii), G(ii)), merged image (A(iii),B(iii),C(iii),D(iii),D(iv),E(iii),F(iii),G(iii),G(iv)). Magenta colour depicts autofluorescence. Scale bars:50µm, n=sample number.

These data suggest that the progenitor cells are mitotically active and that their mitotic activity correlates with the eventual fate of the progenitor.

III.2.4. Expression dynamics of shoot stem cell regulator, WUS in regenerating progenitor

The shoot stem cell regulator, WUSCHEL (WUS), is crucial for maintaining the structural and functional integrity of shoot and floral meristems during development. The *wus* loss-of-function mutant produces defective shoot meristems that terminate prematurely in an aberrant flat structure (Laux *et al.*, 1996). The phenotype is more pronounced in tissue culture, where shoot regeneration is completely abolished in the *wus* mutant (Chatfield *et al.*, 2013; Zhang *et al.*, 2017). Given the importance of WUS in forming functional shoot meristems, I questioned whether pseudo-progenitors are non-productive due to the absence of WUS.

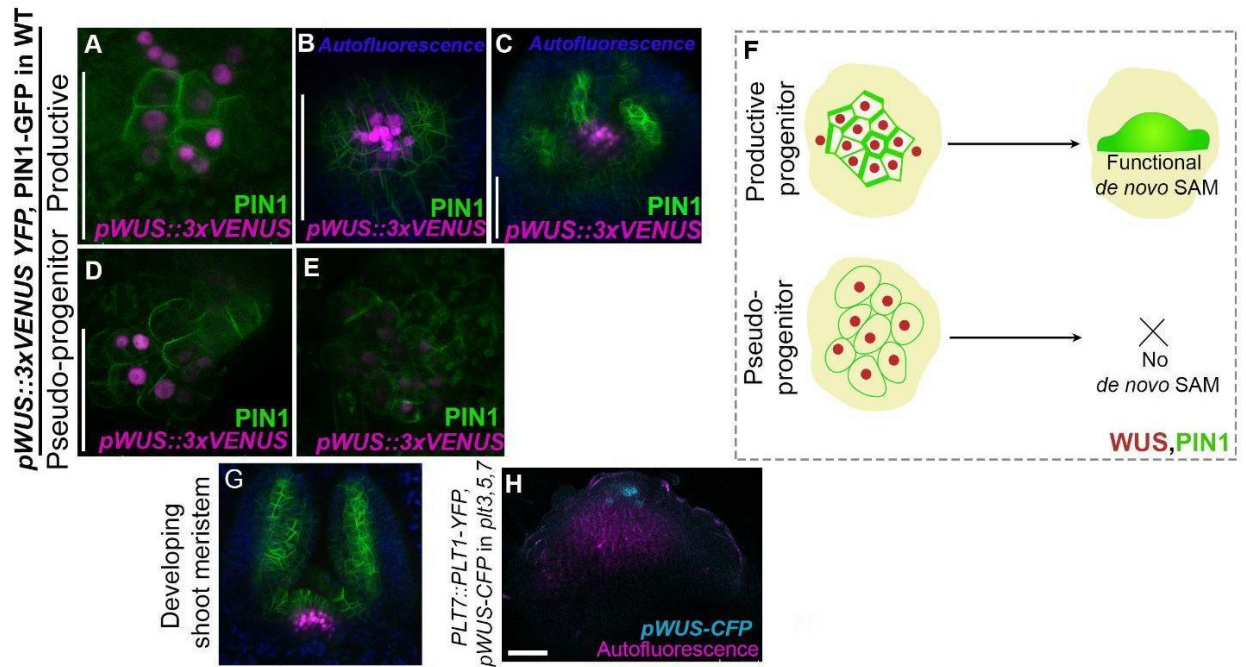


Figure. 3.7: Abundance of shoot stem cell regulators do not guarantee productive-fate during *de novo* shoot organogenesis. (A-E) Representative live image of *pWUS::3XVENUS:tWUS*, *pPIN1::PIN1-GFP* in wildtype showing the WUSCHEL(magenta) activity during various stages of productive progenitor(n=14) (A-C) and pseudo-progenitor(n=26) (D-E). Note that upon progression of a productive progenitor, *pWUS::3XVENUS:tWUS* got confined to central PIN1 marked cells (green) in a promeristem (B) and in a mature shoot (C). The WUS expression vanishes from the pseudo-progenitor as soon as a PIN1-GFP disappears, marking progenitor abortion (E). The images show that the abundance of shoot stem cell regulators does not guarantee productive fate to progenitors. (F) Schematic representation of WUS-3XVENUS pattern in the absence and presence of embryonic positional cues. (G) Representative confocal image of a normal developing SAM expressing PIN1-GFP and WUS-YFP. (H) Representative live image of PLT7::PLT1-YFP, *pWUS-erCFP* in *plt3,5,7* mutant showing WUSCHEL(cyan) in a SIM callus emitting chlorophyll autofluorescence (magenta) (n=25). GFP channel (A-J,H,K), GFP/YFP merged images (M-Q). 10µm (L), 20µm (A-E), 50µm (F-K, M-Q, S), n=sample number.

Using the *pWUS::3XVENUS-tWUS* construct, driven by a 5.3 kb promoter previously shown to report WUS activity only in progenitors (Zhang et al., 2017), I tracked WUS expression dynamics in the *pPIN1::gPIN1-GFP* Wildtype regenerating callus (Figure 3.7.A - 3.7.F). Interestingly, WUS was expressed in PIN1-GFP-marked cells of both productive and pseudo-progenitors, suggesting that the presence of WUS alone is not an

indicator of a productive fate at the progenitor level (Figures 3.7.A, 3.7.D). As productive progenitors progressed to pro-meristems, WUS expression became confined to and robustly expressed in the central zone (Figure 3.7.B). Upon further progression to shoot meristem, WUS expression recapitulated the pattern seen in a developing Wildtype shoot meristem (Figure 3.7.C, 3.7.G). In contrast, pseudo-progenitors, despite initially having abundant WUS expression, gradually lost this expression. Ultimately, WUS expression disappeared along with PIN1-GFP as the progenitor aborted (Figure 3.7.E). Cells expressing WUS almost always overlapped with PIN1-GFP-marked cells, though there were rare instances where WUS was expressed in random callus cells that neither acquired PIN1-GFP nor underwent organogenesis, but became ball-like structures and continued to retain WUS expression.

Notably, detection of WUS in a genetically engineered background incapable of producing productive progenitors (*pPLT7::cPLT1-vYFP* in *plt3,plt5-2,plt7* mutant) further substantiated the conclusion that expression of the stem cell regulator WUSCHEL alone is not a good predictor of progenitor fate.

III.3. DISCUSSION

The totipotent nature of plant cells underpins the various tissue-culture-based regeneration techniques that allow for the generation of complete organs and organisms from somatic cells. This unique capability is exemplified in the process of *de novo* shoot regeneration from callus, which I utilized as a model system to delve into the mechanisms of cellular self-organization. At the core of my inquiry lies a fundamental question: how do shoots arise from a callus, which is essentially an unorganized mass of cells?

There are clusters of select few cells in the callus that acquire PIN1 localization to the plasma membrane, termed shoot progenitors. These shoot progenitors are the ones that later progress into shoot meristems. My study revealed that not all progenitors formed during *de novo* shoot regeneration are destined to become functional shoot meristems. Rather, they can be broadly classified into two subpopulations: productive progenitors, which successfully form shoot meristems, and pseudo-progenitors, which fail to do so. I tested the expression patterns of several relevant proteins to distinguish productive progenitors from pseudo ones. The shoot stem cell regulator, WUS, is transiently expressed in both productive and pseudo-progenitors and can even be found in non-PIN1-marked callus cells, although rarely. My investigations revealed that the key to distinguishing between these two subpopulations lies not in the abundance of classical shoot stem cell regulators like WUS, but in the expression and localization pattern of polarity proteins, particularly PINFORMED1 (PIN1) and SOSEKI (SOK) proteins. Productive progenitors exhibit a well-organized localization pattern of PIN1 on the cell membrane, which correlates with their ability to progress into shoot meristems. In contrast, pseudo-progenitors display aberrant PIN1 localization. Among the SOK proteins, SOK2 showed a distinct correlation with productive progenitors, being exclusively expressed in their constituent PIN1-GFP-marked cells. Yet another way to distinguish productive progenitors from pseudo-progenitors is their mitotic activity. Productive progenitors displayed higher mitotic activity compared to their pseudo counterparts, with the former showing increased CYCB1;1 expression throughout their progression. Simply put, only those progenitors that sustain the optimal PIN1

localization pattern and heightened mitotic activity continue expressing WUS, while others, which lose this pattern, fail to maintain WUS expression and abort their development (Varapparambath and Mathew *et al.*, 2022).

What is the functional relevance of the PIN1 localization pattern? Proper localization of PIN1 is not just a marker of productive progenitors but also plays an active role in shaping the local biochemical environment necessary for shoot formation. Typically, callus formation occurs in an auxin-rich environment, resulting in cells that predominantly express root-specific genes (Sugimoto *et al.*, 2010; Kareem *et al.*, 2015; Zhai & Xu, 2021). This reliance on auxin suggests a bias towards root development, indicating that a high auxin concentration is not conducive to shoot formation. In nature, plants have mechanisms to create a low-auxin environment that facilitates shoot development. For example, during embryogenesis, cotyledon primordia flanking the developing shoot apical meristem (SAM) effectively draw auxin away, establishing a low auxin environment that promotes functional SAM (Heisler *et al.*, 2005; Möller & Weijers, 2009). A similar phenomenon occurs during axillary meristem development, where the subtending leaf aids in auxin drainage from the axil (Tian & Jiao, 2015). Intriguingly, one could speculate that callus tissue also creates a system for auxin drainage; yet how does this occur? Unlike the SAM or axillary meristem, callus lacks defined structures or organs to facilitate auxin drainage. Thus, understanding how callus cells self-organize to generate regions with low auxin necessary for shoot meristem formation becomes imperative. In this context, the role of the PIN1 protein is pivotal; as an auxin efflux carrier, it localizes to the plasma membrane of the shoot progenitor

cells. These PIN1-marked progenitor cells self-organize to form a localization pattern that effectively facilitates auxin efflux to surrounding cells, creating a conducive environment for shoot meristem development (Varapparambath and Mathew *et al.*, 2022). However, what happens if these PIN1-marked cells fail to self-organize appropriately? Such a failure leads to auxin accumulation, resulting in progenitors that do not progress to shoot meristems—the pseudo-progenitors (Varapparambath and Mathew *et al.*, 2022). This raises an essential point: how does the maintenance of low auxin levels influence progenitor progression? Optimal auxin levels are crucial for sustaining shoot stem cell regulators, including WUSCHEL (WUS) and SHOOT MERISTEMLESS (STM) (Clark *et al.*, 1996; Long *et al.*, 1996; Laux *et al.*, 1996; Gordon *et al.*, 2007; Landrein *et al.*, 2015; Zhang *et al.*, 2017; Ma *et al.*, 2019). Considering the impact of auxin levels on shoot stem cell regulators, it is not surprising that disruption in PIN1 localization could hinder the sustenance of shoot stem cell regulators. Corroborating this notion, cases where PIN1 localization is disrupted, WUS expression diminishes, hinting that proper auxin distribution is necessary to sustain these regulators and drive progenitor progression.

In a nutshell, the mere presence of shoot-promoting factors like WUS is not a good indicator of a progenitor's fate. The key discovery in my research is that the localization pattern of polar proteins on the cell membrane determines whether the progenitors will progress into functional shoot meristems. This finding aligns with the broader understanding of cell polarity as a regulator of fate decisions and spatial organization in plant tissue regeneration.

Chapter IV: Cell wall loosening enzyme XTH9 promotes the cell polarity during de novo shoot regeneration

This work is a part of the published manuscript:

Varapparambath, V.*, **Mathew, M.M.*†**, Shanmukhan, A.P.*, Radhakrishnan, D., Kareem, A., Verma, S., Ramalho, J.J., Manoj, B., Vellandath, A.R., Aiyaz, M., Radha, R.K., Landge, A.N., Mahonen, A.P., Heisler, M.G., Weijers, D., Prasad, K†. (2022). Mechanical conflict caused by a cell-wall-loosening enzyme activates *de novo* shoot regeneration. **Developmental Cell**, 57(17), pp.2063-2080. doi: 10.1016/j.devcel.2022.07.017.

(* Equal contribution, †Co-corresponding author)

IV.1 INTRODUCTION

*"Nothing endures but change."
— Heraclitus.*

In the previous chapter (*chapter 3*), I explored the intricate process by which a select few callus cells, known as shoot progenitors, grow and self-organize into a shoot meristem that ultimately gives rise to a functional shoot. This self-organization hinges on the precise localization patterns of polar proteins such as PIN1 and SOK2, which ensure the productive fate of these progenitors. However, a critical question remains: what maintains the localization pattern of these polarity proteins during *de novo* shoot regeneration?

This chapter aims to address this question by delving deeper into the cellular environment of the progenitors and their surrounding cells. Central to this investigation is the enzyme XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9), a member of the XTH family of cell wall-modifying proteins known for their role in cell wall loosening (Nishitani & Vissenberg, 2006; Van Sandt *et al.*, 2007). XTH enzymes modify cell wall structure by cutting and rejoining load-bearing cross-links between cellulose microfibrils, a mechanism that impacts plant growth and development (Fry *et al.*, 1992). XTH9 expression has been reported to coordinate with various developmental stages, including differentiation from vegetative and floral meristems, and even cell elongation in inflorescence stems and roots.

By using a combination of genetic and live-imaging approaches, this chapter investigates how XTH9 activity drives the progression of progenitors into functional shoot meristems. My research reveals that XTH9 exhibits a distinct expression pattern that is

transcriptionally regulated by the CUP-SHAPED COTYLEDON 2 (CUC2) transcription factor. CUC2 is well-known for its role in defining organ boundaries during shoot meristem formation, and it has also been identified as a crucial promoter of shoot regeneration (Gordon et al., 2007; Kareem et al., 2015; Varapparambath & Mathew, 2022). During *de novo* shoot regeneration, CUC2 specifically regulates and confines XTH9 expression to a shell of cells surrounding the progenitors. My findings suggest that XTH9 plays a non-cell-autonomous role in shoot progenitor development—facilitating the expansion of surrounding cells to support the growth and polar localization of PIN1 within the progenitors themselves.

IV.2 RESULTS

IV.2.1. XTH9 is expressed in a shell of cells encapsulating the shoot progenitor

In the previous chapter, I discussed the discovery of two distinct types of progenitors: productive progenitors and pseudo-progenitors. The key features distinguishing productive progenitors from pseudo ones include the localization patterns of polarity proteins and the compact arrangement of the progenitor cells (Figure 3.1D-3.1K). This discovery raises an important question: what maintains the proper localization of these polarity proteins and the compact organization of progenitor cells?

A genome-wide comparative transcriptome analysis between genetically engineered backgrounds that are either capable or incapable of successful shoot regeneration, followed by Gene Ontology (GO) analysis, revealed an enrichment of XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9) in the backgrounds capable of shoot

regeneration (Varapparambath and Mathew et al., 2022). *XTH9* belongs to the XTH genes family, known for causing cell wall loosening (Fry *et al.*, 1992; Van Sandt *et al.*, 2007). *XTH9* activity has been shown to promote cell elongation in roots (Hyodo *et al.*, 2003). Given that it is the pseudo-progenitors that have expanded, loosened and bubble shaped cells, it was intriguing that the expression of the cell wall-loosening function of *XTH9*, was elevated in background producing an increased number of productive shoot progenitors. This contradiction warranted further investigation.

To explore this, I tracked the expression dynamics of *XTH9* in both productive and pseudo-progenitors and discovered a striking expression pattern (Figure 4.1). In productive progenitors, *XTH9* was found exclusively outside the PIN1-GFP-marked cells, specifically in the 1–2 cell layers immediately encircling the progenitor (Figure 4.1.A- 4.1.F). A 3D reconstruction of confocal images from productive progenitors at the 3–4 cell stage revealed that a shell of *XTH9*-expressing cells encapsulates the PIN1- GFP-marked, sphere-like progenitor (Figure 4.1.J - 4.1.K). The earliest detection of this distinct pattern—where PIN1-GFP and *XTH9*-YFP domains were mutually exclusive—was at the 2–3 cell stage of progenitors (Figure 4.1.B). An optical section through the middle plane of 20–30 celled productive progenitors showcased a clear spatial demarcation: a disc of PIN1-GFP-marked cells were surrounded by a ring of *XTH9*-marked cells (Figure 4.1.G.iv- 4.1.I.v). However, an optical section through the top layer showed only *XTH9*-expressing cells (Figure 4.1.G.ii- 4.1.G.iii). This detailed expression analysis reaffirms that *XTH9*-expressing cells encapsulate the PIN1-marked cells of productive progenitors (4.1.K). This expression pattern persists throughout progenitor progression until the promeristem stage—a dome-

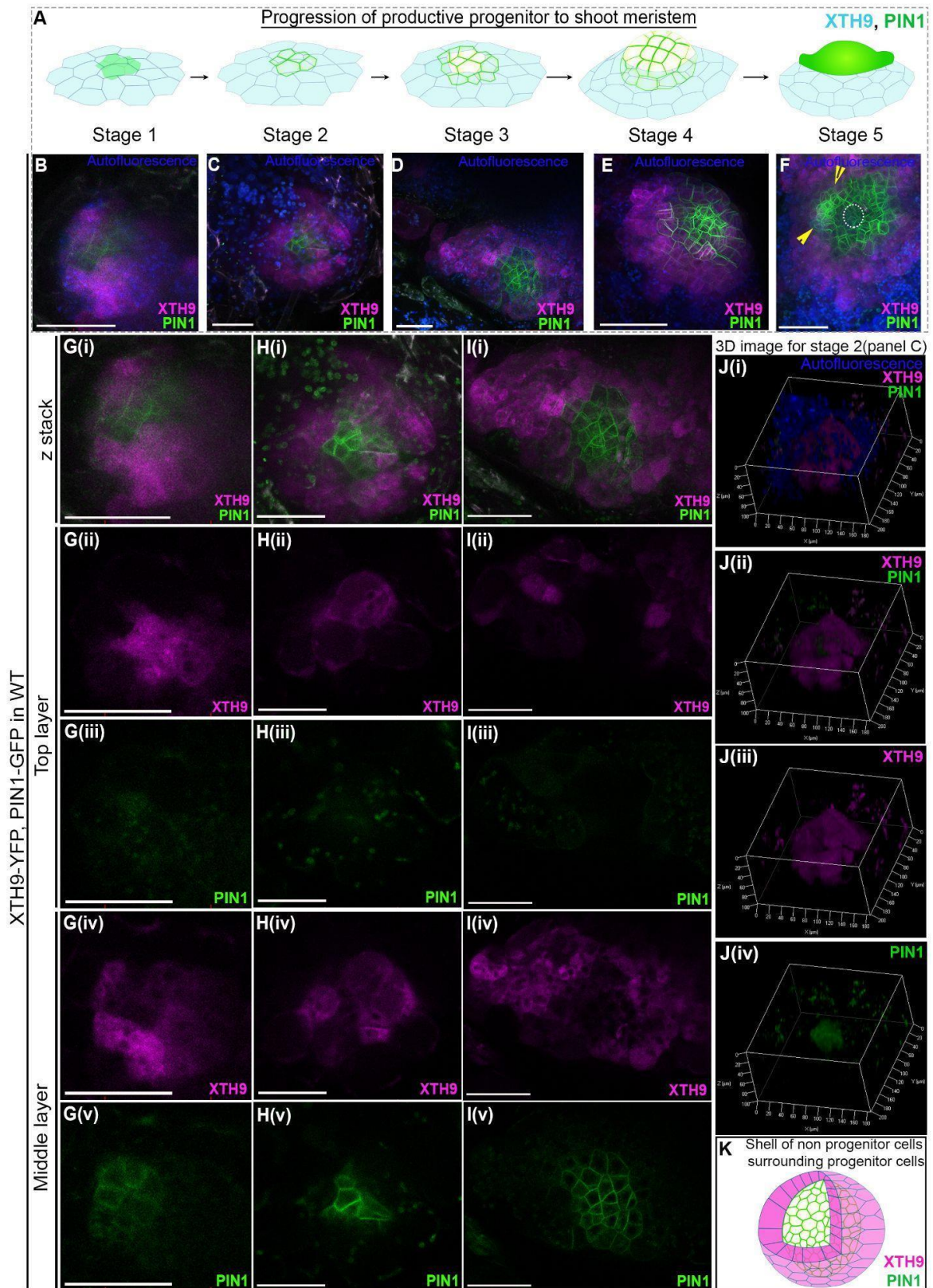


Figure. 4.1: XTH9, a cell wall loosening enzyme promotes the self-organization of progenitor cells non-cell autonomously. (A) Schematic depicting XTH9 expression during progenitor progression. (B-F) Representative live-images showing XTH9-YFP expression at different times points of productive progenitor progression. B, C, D, E and F corresponds to stages 1, 2, 3, 4 and 5 respectively. Till stage 2(8-10 celled stage, C) XTH9 is confined to the shell of non-progenitor cells and excluded from the PIN1-GFP marked progenitor (B, C). At stage 3(15-20 celled stage, D), XTH9 is not detectable at the top of the PIN1-marked cells. At stage 4(60-70 celled promeristem stage, E), the PIN1 marked cells are seen emerging as a dome-shaped structure through the overlaying XTH9 layer of cells. By stage 5(shoot meristem, F) XTH9 is detected at the peripheral cells of mature meristem, and at the base of emerging leaf primordia (yellow arrowheads), but excluded from meristem centre (white dotted circle) (F). (n=18(stage 1), 18(stage 2), 14(stage 3), 30(stage 4), 24(stage 5)). (G-I) Representative live images showing the mutually exclusive domains of PIN1 and XTH9 in the first three stages of developing progenitor, G(i)-G(v) corresponds to B, H(i)-H(v) corresponds to C, I(i)-I(v) corresponds to D. Complete z-stack of XTH9-YFP, PIN1-GFP (in GFP/YFP merged channel G(i), H(i), I(i)). Top layer of z-stack of XTH9-YFP, PIN1-GFP in YFP channel (G(ii), H(ii), I(ii)), in GFP channel (G(iii), H(iii), I(iii)). Middle layer of z-stack of XTH9-YFP, PIN1-GFP in YFP channel (G(iv), H(iv), I(iv)), in GFP channel (G(v), H(v), I(v)). (J) Representative 3D image of z-stack of stage 2 (C). In XTH9-YFP PIN1-GFP line, a shell of XTH9-YFP marked cells encapsulating the PIN1-GFP in marked cells in GFP/YFP/autofluorescence merged channel (J(i)), in the GFP/YFP merge channel(J(ii)), in the YFP channel (J(iii)), in the GFP channel (J(iv)) (White arrowhead:progenitor). Green puncta: chlorophyll autofluorescence. (K) Schematic depicting the XTH9 marked non-progenitor shell, wrapping the PIN1 marked progenitor. Scale bars: 50µm, n:sample number.

shaped stage where PIN1-marked cells emerge through the overlying XTH9-marked cells (stage 4; Figure 4.1, 4.1.E). At this stage, XTH9 becomes confined to the base of the promeristem in a ring-like fashion (Figure 4.1.E). As the promeristem progresses to a functional shoot meristem, XTH9 expression is confined to the base of emerging leaf primordia but stays clear from the meristem (stage 5; Figure 4.1.F).

Unlike productive progenitors, pseudo-progenitors showed overlapping expression domains of PIN1-GFP and XTH9-YFP (Figure 4.2). In pseudo-progenitors, XTH9-YFP was robustly expressed within the PIN1-GFP-marked cells in addition to the surrounding 1–2

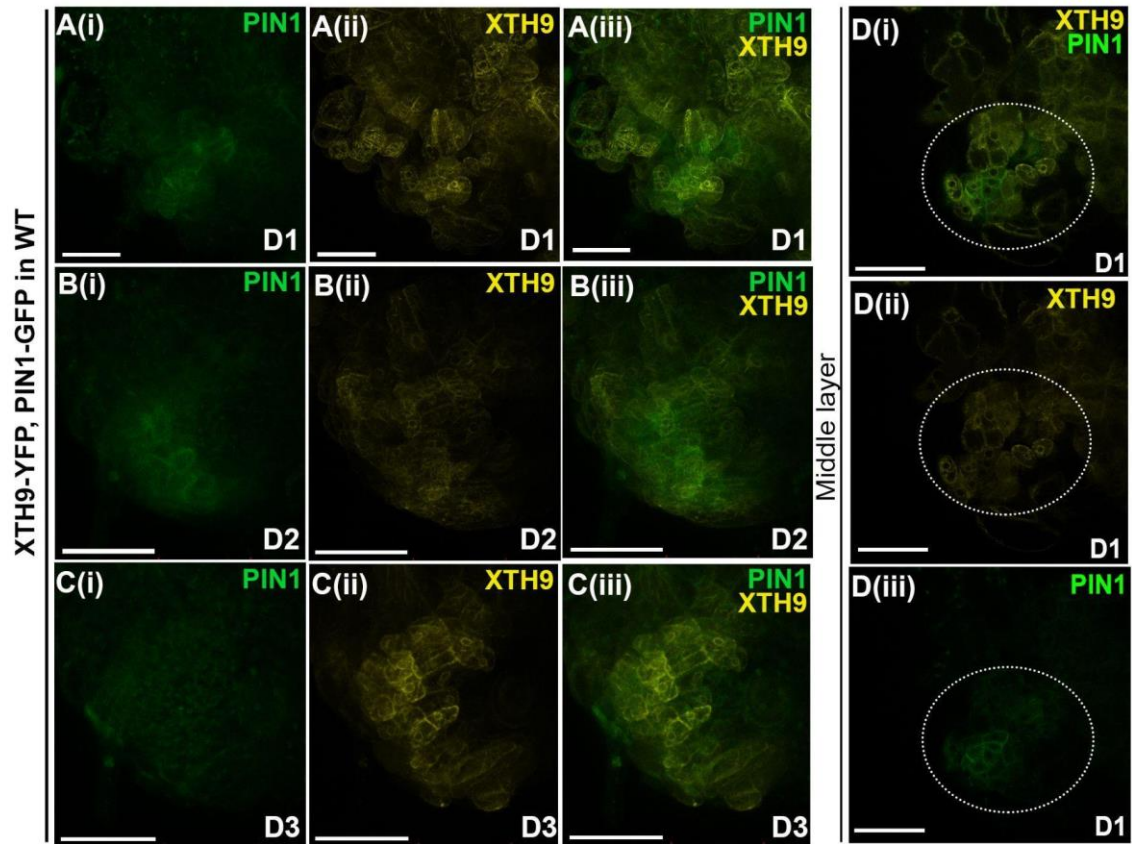


Figure. 4.2: presence of XTH9 within the PIN1-marked progenitor cells impairs the progenitor progression to shoot meristem. (A-D) Representative time lapse images of pseudo-progenitor showing XTH9-YFP(n=20). (K) Middle layer of the pseudo-progenitor. GFP channel (A.i, B.i, C.i, D.i), YFP channel (A.ii, B.ii, C.ii, D.ii), merged image (A.iii, B.iii, C.iii, D.iii) of the pseudo-progenitors with XTH9-YFP, PIN1-GFP in wildtype. D1,D2,D3: Day of spotting the progenitor. Scale bars: 50µm, n=sample number. Error bars represent s.e.m.

cell layers (Figure 4.2). These progenitors do not form compact cell arrangements and eventually abort, as supported by the loss of PIN1-GFP expression (Figure 4.2.C). Thus, it appears that PIN1-marked cells do not tolerate the presence of XTH9, and if XTH9 intrudes into the PIN1 domain, the progenitor loses its PIN1 localization and compact cell arrangement. Collectively, these data suggest that XTH9, when confined to cells surrounding

the progenitor, promotes progenitor progression, but its presence within the progenitor cells disrupts this process.

IV.2.2. XTH9 expression in surrounding cells is essential to promote PIN1 localization to plasma membrane during *de novo* shoot regeneration

With the spatial specificity of XTH9 established, I next explored its functional role in *de novo* shoot regeneration. Towards this, I used the *xth9* (3'UTR) loss of function mutant which during normal development had no aberrant defects in shoot and produced robust shoot derived organs comparable to Wildtype (Varapparambath and Mathew et al., 2022). I asked if removal of XTH9 impinges on the shoot regeneration ability. Indeed, the *xth9* mutant showed dramatic reduction in shoot regeneration compared to Wildtype, revealing that *de novo* shoot regeneration is hypersensitive to *xth9* (Figure 4.3.A- 4.3.C). This impairment in shoot regeneration was not due to defective callus, as neither the callus formation nor the callus phenotype was compromised. This hints at the possibility that shoot regeneration may be perturbed at the progenitor level.

Since XTH9-expressing cells encapsulate the PIN1-marked progenitor cells, I investigated the behavior of these encapsulating cells—referred to as 'non-progenitor cells' for simplicity—in both *xth9* mutants and wild-type plants (Figure 4.3.D-4.3.J). In wild-type, non-progenitor cells surrounding productive progenitors (20–25 celled stage) increased their surface area by 12.76% over 5 hours and 30 minutes. In contrast, non-progenitor cells in *xth9* mutants expanded by only 5.17%, a 2.47-fold reduction (Figure 4.3.D). This reveals that the expansion of non-progenitor cells allows progenitor cells to grow, maintain PIN1

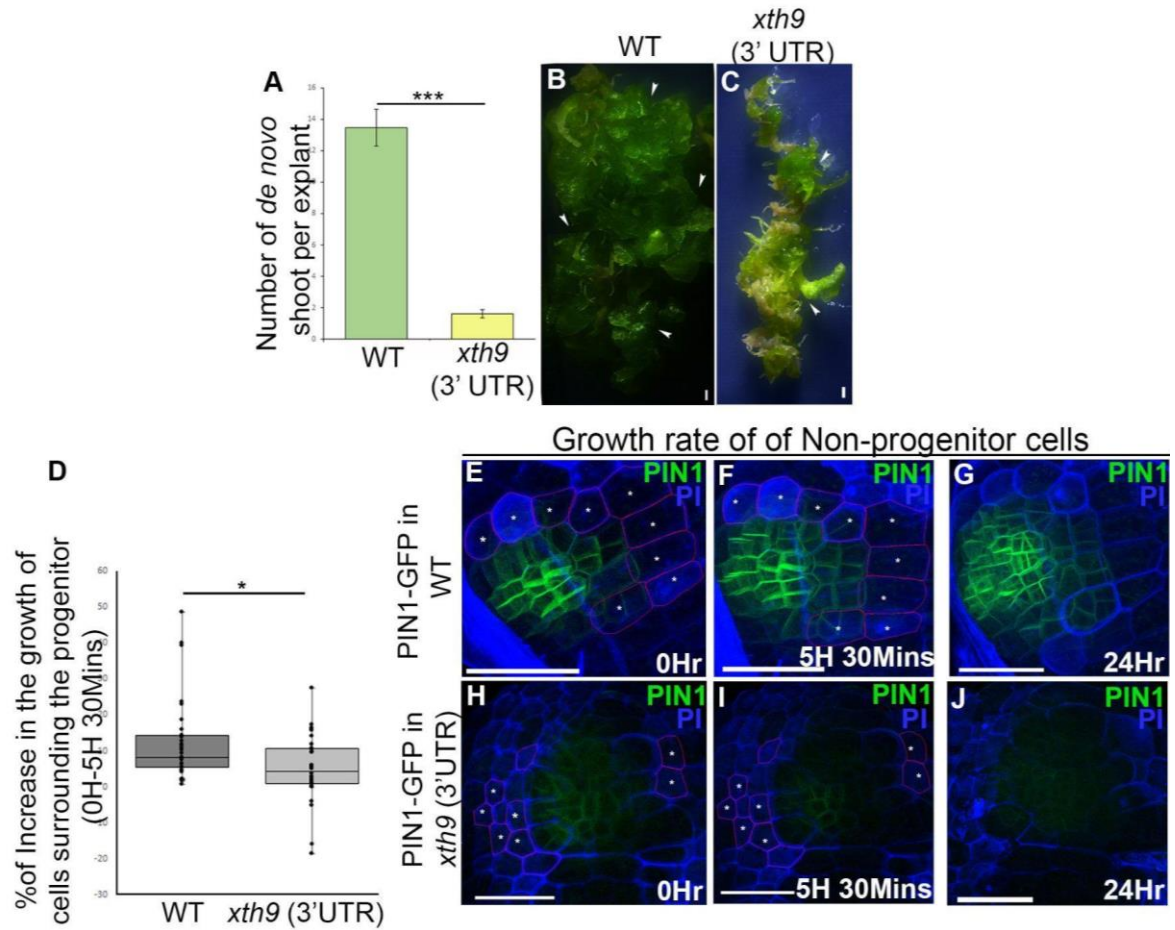


Figure. 4.3: Activity of XTH9 is essential for shoot meristem formation *de novo*. (A-C) Shoot regeneration in *xth9* (n=105, 1.602 ± 0.259 , ***P=0.0007686, Welch Two Sample t-test) compared to wildtype (n=77 13.463 ± 1.160). (D) Graph depicting the percentage increase in cell surface area of non-progenitor cells of wildtype compared to *xth9* over 5.30Hr (n=22, *P=0.035809, Mann-Whitney U test). (E-J) Representative time-lapse images of productive progenitor (20-25 celled) in wildtype (E-G) (n=32), and progenitors (20-25 celled) in *xth9* (n=12) (H-J) at 0Hr, 5Hr 30mins, and 24Hr. White asterisks: Cells tracked. White arrowheads: Regenerated shoots. Error bar: s.e.m. Scale bars: 50μm (E-J), 1mm (B-C), n: sample number.

localization, and progress to shoot meristem. When non-progenitor cell expansion is restricted, the progenitor cells stop growing, lose PIN1 localization, and abort (Figure 4.3.H-

4.3.J). Thus, XTH9 is critical in non-progenitor cells for maintaining PIN1 localization and promoting progenitor progression non-cell autonomously.

IV.2.3. Specificity in transcriptional regulation of XTH9 is necessary for successful shoot regeneration

I next asked what regulates the spatial specific expression of XTH9- confining XTH9 only to the non-progenitor cells encapsulating the progenitor? It was revealed that XTH9 is under the transcriptional control of a shoot promoting Transcription Factor, CUC2(CUP-SHAPED COTYLEDON 2). CUC2 was shown to bind to the intragenic elements of XTH9 and activate its transcription (Varapparambath and Mathew et al., 2022). Notably, I found that the expression domain of CUC2 overlaps with that of XTH9: CUC2 is expressed only in the non-progenitor cells encapsulating the progenitor and was excluded from the PIN1-GFP marked cells (Figure 4.4A- 4.4.F). Further supporting the regulation of XTH9 by CUC2, it was found that dramatic reduction in shoot regeneration in *cuc2-3* loss of function mutant was partly rescued by complementing functional *XTH9* under CUC2 promoter (*pCUC2::gXTH9-YFP* in *cuc2-3*) (Varapparambath and Mathew et al., 2022). Live imaging of the progenitors in *cuc2-3* (*pPIN1::gPIN1-GFP* in *cuc2-3*) revealed that the mutant exhibited a sharp decline in progenitor formation, and the progenitors that rarely form are the ones after prolonged SIM (shoot induction medium) incubation (Figure 4.4.G). These progenitors, however, resembled pseudo progenitors with PIN1 delocalized from the plasma membrane of their constituent cells and fail to progress into shoot meristem (Figure 4.4.H). This data shows that CUC2 acts through XTH9 to promote meristem formation, and removal

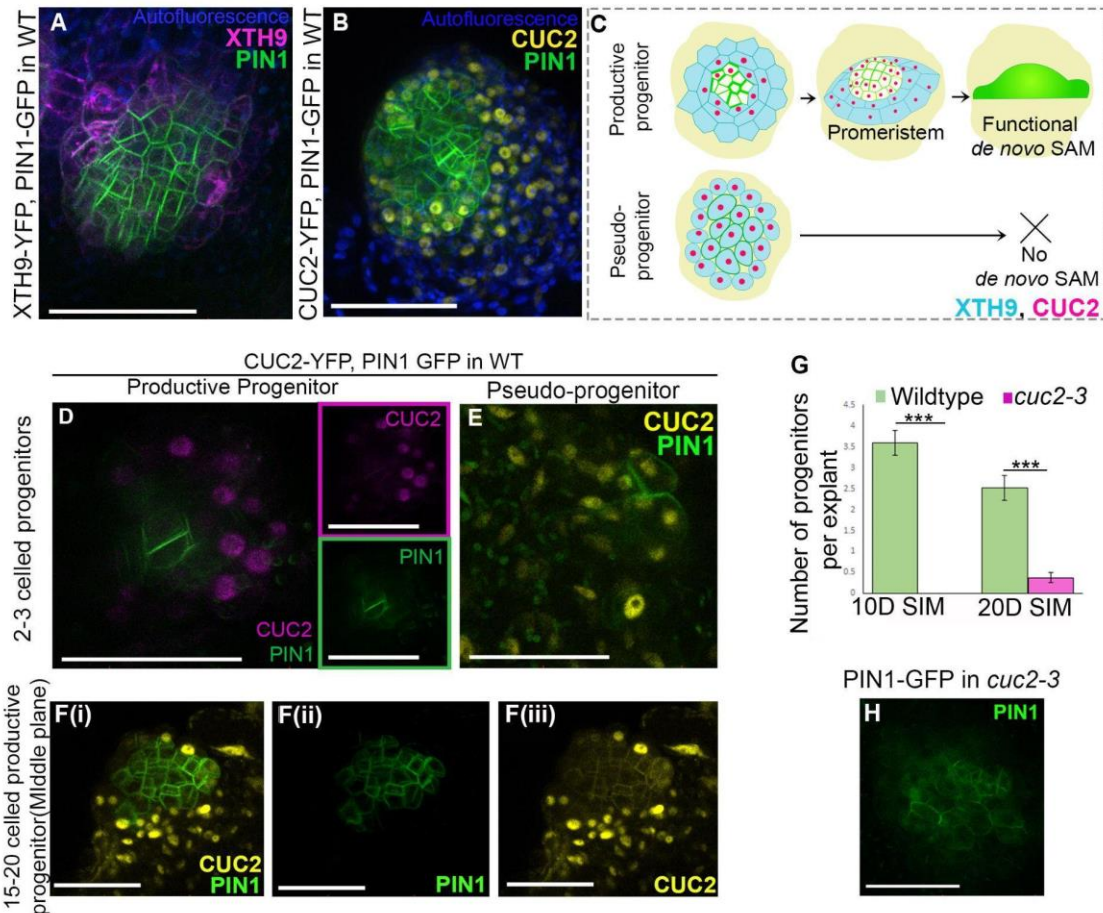


Figure. 4.4: CUC2 is essential for progenitor formation and promotes progenitor progression non-cell autonomously. (A-B) Representative live images for XTH9 and CUC2 expression in wildtype. (C) Schematic depicting CUC2 and XTH9 expression in productive progenitor and pseudo-progenitor during shoot regeneration. (D-F) Representative live images of productive progenitors and pseudo-progenitor showing CUC2 expression. CUC2 (magenta) is excluded from the PIN1-marked productive progenitor cells and rather forms a ring around it at 2-3 celled stage (D) or even at 15-20 celled stage (F). CUC2 is present within the PIN1-marked cells of pseudo-progenitor (E). Merge channels F.i, GFP channel F.ii, YFP channel F.iii. (G) Graph depicting the number of progenitors in *cuc2-3* single mutant compared to wildtype during the 10th day of SIM incubation (wildtype; n=112, *cuc2-3*; n=216, ***P=1.009e-08, Welch Two Sample t-test) and 20th day (wildtype; n=92, *cuc2-3*; n=114, ***P=6.248e-05, Welch's Two Sample t-test) of SIM incubation. (H) Representative images showing PIN1 expression in the progenitor of *cuc2-3*. Error bar:s.e.m. Scale bars:50μm, n:sample number.

of either CUC2 or XTH9 impairs the PIN1 localization pattern of the progenitors rendering them unproductive.

Given that CUC1, another member of the CUP-SHAPED COTYLEDON family, is also known to enhance shoot regeneration through ESR (Iwase et al., 2017), I questioned whether CUC1 might similarly regulate the spatial specific activity of XTH9. This is important because CUC1 has been shown to act redundantly with CUC2 (Hibara *et al.*, 2006). However, my findings revealed that unlike CUC2, the expression domain of CUC1 did not overlap with that of XTH9. Instead, CUC1 was strongly expressed within the progenitor cells themselves, and not in the surrounding non-progenitor cells (Figure 4.5.A - 4.5.F). Furthermore, the progenitors in *cuc1-5* mutants (*pPIN1::gPIN1-GFP* in *cuc1-5*) were comparable to the wild-type productive progenitors, with compactly arranged cells and robust PIN1-GFP localization at the plasma membrane (Figure 4.5.G - 4.5.K). These progenitors successfully self-organized into dome-shaped pro-meristems and eventually functional shoot meristems (Figure 4.5.K).

Together, these findings suggest that the spatial specific activity of *XTH9* gene is regulated by CUC2, but not its redundant gene, CUC1. The data highlight the necessity of the CUC2-XTH9 regulatory axis for shoot regeneration, and shows that the axis operates in non-progenitor cells conferring productive fate to regenerating shoot progenitors, non-cell autonomously.

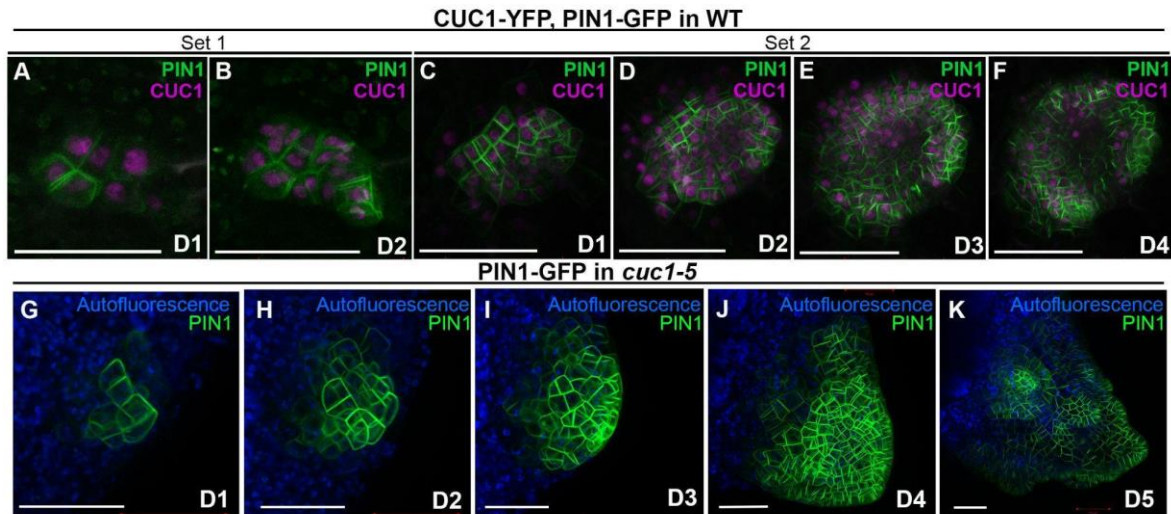


Figure. 4.5: CUC1 is dispensable for *de novo* shoot regeneration. (A-F) Representative combined time lapse images showing the expression pattern of *pCUC1::gCUC1-vYFP*, *pPIN1::PIN1-GFP* in a progressing progenitor, which revealed that CUC1 do not have heterogeneous expression. CUC1-YFP (magenta) expression can be observed in all the PIN1-GFP (green) expressing progenitor cells right from 6-cell stages (n=13). (A-F) shows the GFP/YFP merged channel for *pCUC1::CUC1-vYFP*, *pPIN1::gPIN1-GFP* in wildtype. (G-K) Representative time lapse images of PIN1-GFP (green) in *cuc1-5* single mutant, showing that progenitors can progress into functional shoot meristem in the absence of CUC1 (n=7). These progenitors do not display any defects in the PIN1 localization pattern. (G-K) shows the GFP/autofluorescence merged channel for PIN1-GFP in *cuc1-5* mutant. Scale bars: 50µm. n=sample number.

IV.3. DISCUSSION

The previous chapter focuses on how important it is for the shoot progenitors to have proper PIN1 localization and compact arrangement of their cells for successful shoot regeneration. In this chapter, I deal with what facilitates the required PIN1 localization pattern cell arrangement. I discovered that the factor responsible is XTH9 and its expression in a specific domain: exclusively in a shell of cells encapsulating the progenitor. The exclusion of XTH9 activity from the progenitors themselves is vital, plausibly to ensure precise spatially

regulated cell expansion. This spatial specific activity of XTH9 gene is transcriptionally regulated by the shoot promoting factor CUC2 whose expression domain overlaps with that of XTH9. The CUC2-XTH9 regulatory axis is specific to the non-progenitor cells and necessary for successful progenitor progression. What makes this regulatory axis particularly interesting is its non-cell-autonomous mode of operation. Activity of the XTH9-CUC2 axis in the surrounding non-progenitor cells influences the behavior of progenitors by promoting the robust localization of PIN1 in progenitor cells. Perturbing this axis by mutations in either CUC2 or XTH9 impairs the PIN1 localization pattern rendering the progenitors unsuccessful. Interestingly, CUC1 which acts redundantly with CUC2 in developmental context do not play a role in the spatial specific activity of XTH9 gene during *de novo* shoot regeneration. Nevertheless, one cannot discount the possibility of CUC1 regulating other XTH genes. Reasoning that progenitor cells rapidly divide, it is likely that the wall remodeling during their division is facilitated by other XTH genes whose expression domain overlap with that of CUC1.

Through this chapter I demonstrate that the CUC2-XTH9 regulatory axis operates in neighboring non-progenitor cells to confer a productive fate to regenerating progenitors non-cell-autonomously, with the cell wall loosening enzyme XTH9 promoting polar localization of PIN1 during *de novo* shoot regeneration. Given the specific role of CUC2 in regulating XTH9 expression, future studies could delve into how CUC2 might interact with other regulatory networks involved in shoot progenitor maintenance. Exploring how CUC2 coordinates with other signaling pathways or transcription factors that influence cell wall

properties could reveal additional mechanisms supporting tissue organization and successful progenitor progression in shoot regeneration.

Chapter V: Mechanical conflict between neighboring cells activates de novo shoot regeneration

This work is a part of the published manuscript:

Varapparambath, V.*, **Mathew, M.M.*†**, Shanmukhan, A.P.*, Radhakrishnan, D., Kareem, A., Verma, S., Ramalho, J.J., Manoj, B., Vellandath, A.R., Aiyaz, M., Radha, R.K., Landge, A.N., Mahonen, A.P., Heisler, M.G., Weijers, D., Prasad, K†. (2022). Mechanical conflict caused by a cell-wall-loosening enzyme activates *de novo* shoot regeneration. **Developmental Cell**, 57(17), pp.2063-2080. doi: 10.1016/j.devcel.2022.07.017.

(* *Equal contribution*, †*Co-corresponding author*)

V.1 INTRODUCTION

"Conflict is the beginning of consciousness."

— *M. Esther Harding*

Multicellular organ formation results from the spatial and temporal organization of multiple cell types. These organs acquire specific shapes critical for specialized functions through a process known as morphogenesis. Most of our current understanding of morphogenesis, in both plants and animals, stems from a biochemical perspective (Wartlick *et al.*, 2011; Kicheva & Briscoe, 2023). However, it has become increasingly clear that biochemical signals alone cannot fully explain the process. Research over the years has strongly emphasized that, in addition to biochemical cues, mechanical forces play a significant role in development (LeGoff & Lecuit, 2015). As a result, tissue mechanics have been incorporated into various models to account for observations that biochemical signals could not explain alone (Aegerter-Wilmsen *et al.*, 2007; Hufnagel *et al.*, 2007; Egginton, 2011; Uyttewaal *et al.*, 2012). This idea is not new: a century ago, D'Arcy Thompson proposed that biological systems are shaped by physical constraints (Thomson, 1917). Later, Trinkaus expanded on this idea, stating that morphogenesis can only be fully understood through an integrative approach, considering genetic factors, molecular structures, cellular interactions, and the physical environment (Trinkaus, 1984).

In plants, morphogenesis and development heavily depend on mechanical factors, including turgor pressure (TP), cell wall extensibility, and tissue stress (Beauzamy *et al.*,

2015). The intrinsic mechanical forces within plant cells arise from their ability to regulate hydrostatic pressure—turgor pressure. Various factors influence TP, such as developmental stage, environmental conditions, cell size, and local biological factors (Beauzamy *et al.*, 2014; Long *et al.*, 2020). Plant cells are under high TP, and cell expansion can only occur if cells remain under pressure (Murray *et al.*, 2012). This is crucial, as cell expansion is the first step toward cell growth and division, both of which are fundamental to morphogenesis. When tensile forces exceed a certain threshold, cell walls undergo irreversible deformation, referred to as expansion (Mouliia *et al.*, 2015; Cosgrove, 2016). Cell expansion occurs through mechanisms like cell wall acidification (Dale, 1986), activity of enzymes such as expansins (Cosgrove, 1996), pectin-modifying enzymes (Peaucelle *et al.*, 2008, 2011), and XTHs (Varapparambath and Mathew *et al.*, 2022). These processes are linked to cell wall extensibility, or the ability of the cell wall to stretch and expand as the cell grows (Cosgrove, 1993, 2024).

Morphogenesis in multicellular tissues depends not only on the mechanical properties of individual cells but also on tissue-level stress. Variations in the mechanical properties or growth rates of different tissue layers can generate these stresses (Hejnowicz & Sievers, 1996). For example, the rapidly growing tissues in the plant shoot apical meristem experience compressive stress, while the epidermis is under tensile stress (Hussey, 1971; Green & Poethig, 1982; Hejnowicz & Sievers, 1995). These tissue stresses, whether compressive or tensile, exert additional forces on cells, influencing wall stress as cells with different mechanical properties are interconnected in plants.

How does the tissue stress and wall stress interact with the biochemical properties to bring about morphogenesis? Studies have implicated that mechanical properties could feedback on biochemical properties (Heisler *et al.*, 2010; Dupont *et al.*, 2011; Wada *et al.*, 2011; Landrein *et al.*, 2015), and *vice versa* (Milani *et al.*, 2013; Ali *et al.*, 2014), but warrants further investigations. In this chapter, I investigate the interplay between mechanical and biochemical properties, using *de novo* shoot meristem formation as a model. I discovered that a mechanical conflict between juxtaposed cells, driven by the cell wall-loosening enzyme XTH9, activates cell polarity in progenitor cells and promotes meristem formation.

V.2. RESULTS

V.2.1. Progenitor cells grow faster than its surrounding cells

In Chapter 4, I identified that the cell wall-loosening enzyme, XTH9 is spatially regulated and expressed specifically in the non-progenitor cells surrounding the progenitors (Figure 4.1). Given that the spatio-temporal regulation of genes is known to cause localized differences in cell behavior (Gilmour *et al.*, 2017), I investigated the behavior of regenerating progenitors in relation to their surrounding cells. To assess this, I tracked the progenitors through real-time live imaging at 0 hours, 5 hours 30 minutes, 9 hours, and 24 hours, analyzing their behavior in terms of growth, considering both expansion and division (Figure 5.1). First, I measured the overall growth of progenitors by calculating their 2D surface area (SA). The measurement was performed on confocal stacks, presented as 2D maximum intensity projections (MIPs), with PIN1-GFP marking progenitor cells and PI staining the

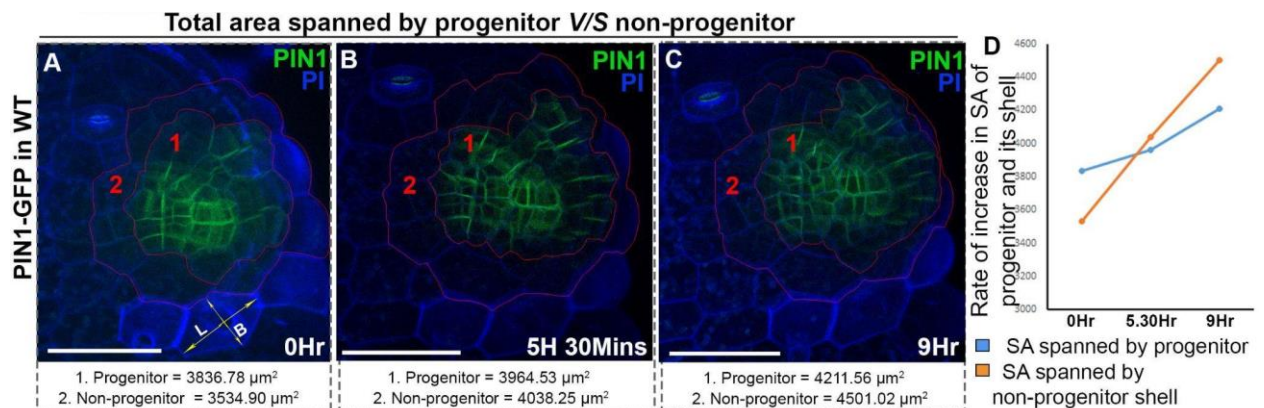


Figure. 5.1: The area spanned by the progenitor increases more than that spanned by the surrounding non-progenitor tissue during progenitor progression. (A-C) Representative time lapse images showing that quantification of the surface area occupied by progenitors at time points 0Hr, 5Hr 30 mins, and 9Hr, revealed that both the progenitor and non-progenitor tissue grow in size over time. Yellow arrows represent Length and Breadth(A). **(D)** Graph depicting the increase in area occupied by the progenitor and the non-progenitor at time points 0hr, 5hr and 9Hr. Scale bars: $50\mu\text{m}$, n=sample number. Error bars represent s.e.m.

surrounding cells. Remarkably, within 9 hours, the area spanned by PIN1-GFP marked progenitor cells increased by 54.85%, while the area spanned by non-progenitor cells showed only a 23.29% increase, highlighting a 2.35-fold faster increase in the SA of the progenitors (Figure 5.1).

Next, I sought to determine whether this increase in growth rate in progenitor cells was due to cell expansion, cell division, or a combination of both. To achieve this, I segmented the cells from time-course confocal stacks (at 0 hours, 2.5 hours, 9 hours, and 24 hours) in MorphoGraphX 2.0 r1-254 (MGX) (Barbier de Reuille *et al.*, 2015; Strauss *et al.*, 2022), using cell outlines visualized by PIN1-GFP or PI staining (Figure 5.2.A - 5.2.L). I then performed a cell lineage analysis to quantify the frequency of cell divisions over a 24-hour period. Interestingly, the results revealed a stark contrast between progenitor and

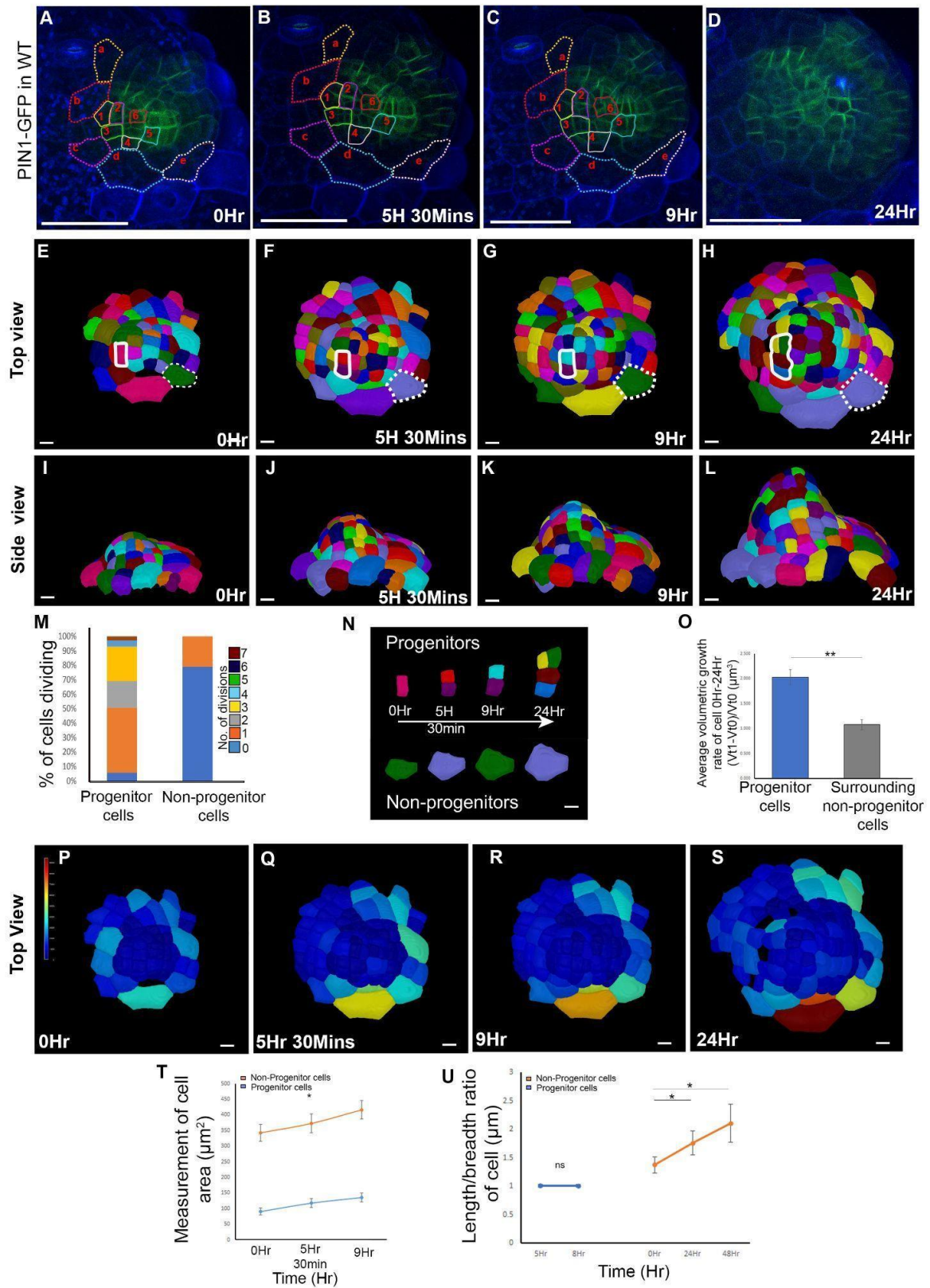


Figure. 5.2 : Progenitor cells are fast growing compared to surrounding non-progenitor cells. (A-D) Representative time-lapse image of wildtype productive progenitor at 0Hr, 5.30Hr, 9Hr, and 24Hr of spotting(n=5). Coloured solid lines and dotted lines enclose clones of progenitor cells and non-progenitor cells respectively. **(E-L)** MGX-based 3D reconstructed model of progenitor in B, through time points 0Hr, 5Hr 30min, 9Hr, and 24Hr. White solid lines and dotted lines in E-H show progenitors and non-progenitors respectively. **(M)** Graph depicting the number of cell divisions in progenitor and surrounding non-progenitor cells in 24Hr. **(N)** MGX-based segmented progenitor and non-progenitor cell lineage from E-H. **(O)** Graph depicting the volumetric increase in progenitor cells and their clones ($x=70$, 2.028 ± 0.14821), and the non-progenitor cells($x=19$, 1.080 ± 0.1012) (** $P=0.0016$, Welch two sample test). **(P-S)** Volumetric heat map (red-high, blue-low); P-S corresponds to E-H. **(T)** Graph depicting the increase in surface area of progenitor cells ($x=21$, (0Hr- 90.5421 ± 11.239) (9Hr- 135.472 ± 14.011)) and non-progenitor cells($x=21$ (0Hr- 342.851 ± 27.389) (9Hr- 416.5 ± 29.86)) at time points 0Hr, 5Hr 30 mins, and 9Hr. (0Hr-9Hr, * $P=0.023176$, Mann-Whitney U test). **(U)** Graph depicting the length/breadth ratio of progenitor ($x=47$, 5Hr(1.013 ± 0.0254) -8Hr(1.0102 ± 0.0209) ns, $P=0.7081$, Two sample paired t test) and non-progenitor cells($x=11$, (0Hr- 1.3741 ± 0.01456)(24Hr- 1.7636 ± 0.2103) (48Hr- 2.1024 ± 0.3319)) (0Hr-24Hr, * $P=0.0074$, Two sample paired t test, 0Hr-48Hr, * $P=0.0151$, Two sample paired t test) over time. Scale bars:50 μ m A-D), Scale bars: 10 μ m (E-L, N,P-S) n=sample number, x=cell number. Error bars represent s.e.m.

surrounding non-progenitor cells. Approximately 95% of progenitor cells underwent division, with some completing up to seven rounds of proliferation. In contrast, only 20% of non-progenitor cells divided, and that too only once, while 80% remained mitotically inactive (Figure 5.2.M). This was further visualized through lineage tracing on confocal stacks and MGX segmentations, revealing that progenitor cells produced several clones, whereas non-progenitor cells did not (Figure 5.2.N). This aligns with previous observations using CYCLIN B1;1 in Chapter 3, which demonstrated that progenitor cells are highly proliferative, while surrounding non-progenitor cells rarely divide (Figure 3.6).

To further assess the growth dynamics, I measured the volumetric change in each cell lineage over 24 hours. This involved comparing the volume of a single cell at 0 hours

with the collective volume of its clones (descendants) at 24 hours. Towards this, volumetric surface meshes of cells and their clones were generated from MGX segmentations of time-course confocal stacks, with each cell lineage manually traced. The cell volumes were then extracted using Cell Analysis 3D/Volume in MGX, and exported for quantification of volumetric growth rates (In collaboration with Dr. João Jacob Ramalho, and Prof. Dolf Weijers, Wageningen University & Research, Netherlands). Results showed that over 24 hours, progenitor cell lineages increased in volume by an average of $2.028 \mu\text{m}^3$, nearly doubling the $1.080 \mu\text{m}^3$ growth observed in surrounding non-progenitor cell lineages during the same time frame (Figure 5.2.O). Next, I measured the volumetric change in each cell over 24 hours. Here also, the cell volumes were then extracted from confocal stacks using Cell Analysis 3D/Volume in MGX, and exported for visualization as heat maps on the segmented meshes. This revealed an interesting finding: at any given time, individual non-progenitor cells have a higher volume than progenitor cells (Figure 5.2.P - 5.2.S). This was further supported by the measured SA of individual progenitor cells and surrounding non-progenitor cells just until their division. It revealed that the increase in SA of individual non-progenitor cells was significantly greater than that of each progenitor cell until division (Figure 5.2.T). Nevertheless, the collective volume of progenitor cell clones is greater than that of non-progenitors.

Together, these results reveal a distinct growth pattern in regenerating calli: progenitor cells primarily grow through cell division, albeit with some expansion, while XTH9 expressing non-progenitor cells grow predominantly by expansion.

V.2.2. Progenitor cells and surrounding non-progenitor cells have different geometries

Given that the PIN1-GFP-marked progenitor cells grow faster than the surrounding XTH9-expressing non-progenitor cells, I set out to investigate what impact this difference in growth rate among neighboring cells might have. When a cell grows faster than those around it, it can create mechanical stress that stretches its neighbors, impacting their geometry (LeGoff & Lecuit, 2015; Dorothee *et al.*, 2022). Moreover, modulation of wall properties can lead to altered cell geometries (Bidhendi & Geitmann, 2016). Reflecting these, I observed that non-progenitor cells encapsulating the rapidly growing progenitors adopt an elongated shape. Over 48 hours, the aspect ratio of these non-progenitor cells—calculated as the length-to-breadth ratio (L/B)—increases significantly, from approximately 1.5 to about 2.5, indicating a 53% increase (Figure 5.2.U). This change suggests that non-progenitor cells expand primarily along one axis, growing anisotropically, or in a single, stretched direction. In contrast, progenitor cells exhibit a markedly different growth pattern. The L/B ratio for progenitor cells and their clones remains steady at approximately 1, indicating that progenitor cells grow uniformly in all directions, or isotropically, without favoring one axis over another (Figure 5.2.U). It's important to note that due to the high proliferation rate of progenitor cells, each cell's L/B ratio can only be measured for a limited period (up to about 8 hours) before it divides and forms new cells. Thus, progenitor cells remain isodiametric, while the surrounding non-progenitor cells display a distinct elongated geometry.

Cell geometry, at its core, is thought to arise from two main influences: intrinsic inheritance or external environmental pressures (Marshall, 2011). The shape of non-progenitor cells appears to align with this theory, as they have elongated geometry while

encapsulating the fast-growing progenitor. On the other hand, the origin of the isodiametric shape in progenitor cells—whether it is inherited or shaped by a different set of factors—remains unexplored.

V.2.3. Progenitor cells and surrounding non-progenitor cells lie in different stress fields

Since it is well known that inhomogeneous growth within a tissue generates mechanical stress, I sought to explore the nature of the stress generated by the rapidly growing progenitor cells compared to the slower-growing non-progenitor cells. Several research shows that cortical microtubules (MTs) orient in response to mechanical stress (Williamson, 1990; Hamant *et al.*, 2008; Landrein *et al.*, 2013; Sampathkumar *et al.*, 2014; Creff *et al.*, 2015; Hervieux *et al.*, 2017). Therefore, to investigate the pattern of stress generated, I monitored the MT orientation in these different cell types using CLIP-ASSOCIATED PROTEIN (CLASP) (*pCLASP::vYFP-CLASP:3AT* in wildtype). CLASP is a MT stabilizing protein which binds the full length of MTs and accumulates at growing plus ends (Ambrose & Wasteneys, 2008). I used CLIP-ASSOCIATED PROTEIN (CLASP). Confocal based live imaging with YFP-CLASP revealed clear distinctions in MT orientation between the progenitor and non-progenitor cells of productive progenitors (Figure 5.3). Interestingly, in non-progenitor cells (n=18) surrounding productive progenitors (30- to 50-celled stages, n=23), MTs exhibited a predominantly anisotropic and circumferential orientation (Figure 5.3.C, 5.3.E - 5.3.F, 5.3.H), while in the inner PIN1-GFP marked progenitor cells, MTs were oriented isotropically (Figure 5.3.C, 5.3.E, 5.3.H). To support this qualitative observation, I quantified MT anisotropy in both progenitor and non-progenitor cells using the Fibril Tool in Fiji/ImageJ. Individual cells from confocal stacks

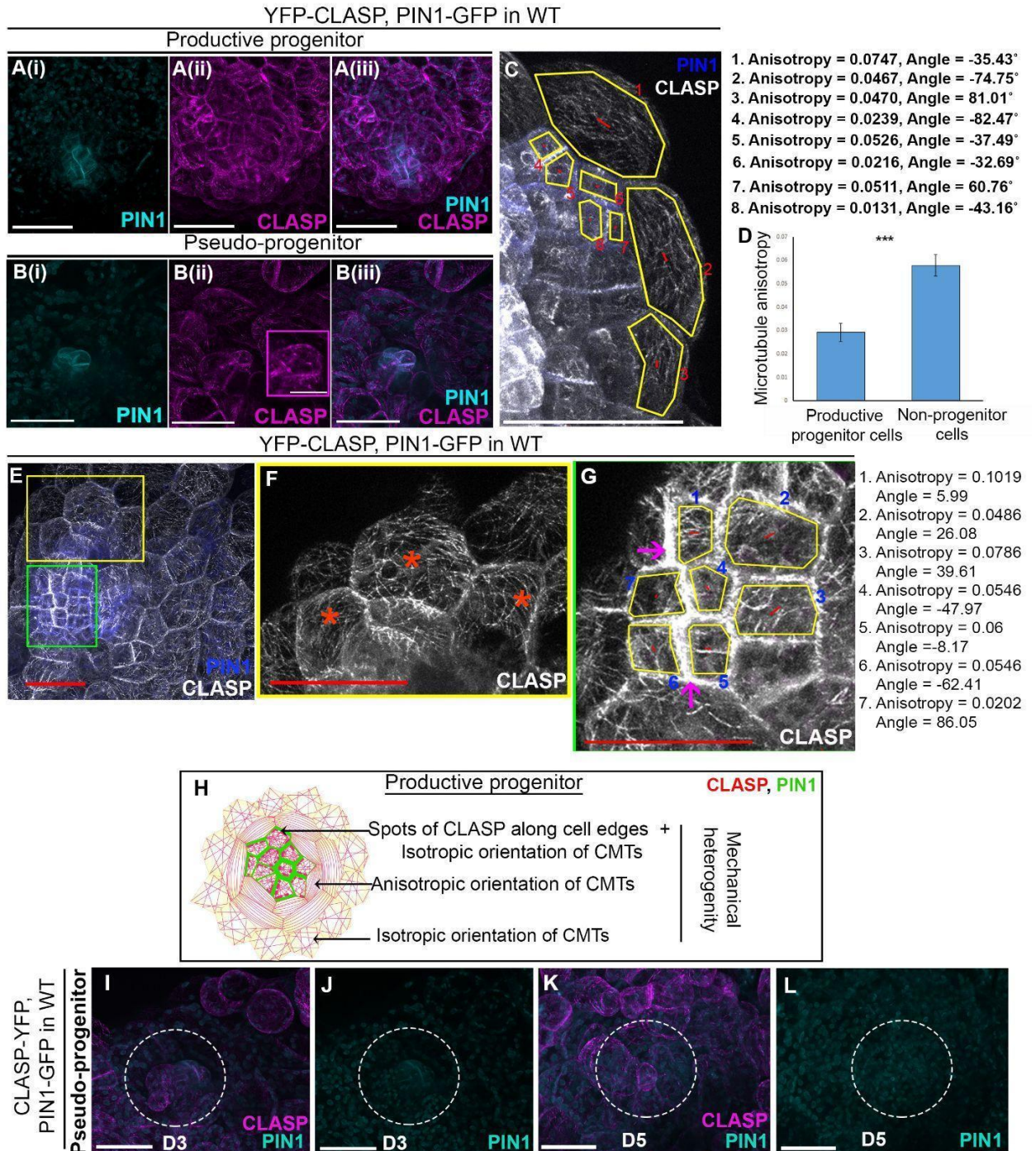


Figure. 5.3: There is a mechanical conflict between the juxtaposed progenitor and non-progenitor cells during *de novo* shoot meristem formation. (A-B) Representative live images of productive and pseudo-progenitors expressing YFP-CLASP. (C) Representative image of a part of 60-celled productive progenitor(n=9) showing CLASP marked MTs in the

non-progenitor cells (1, 2, 3) are anisotropic compared to the progenitor cells (4, 5, 6, 7, 8). **(D)** Graph depicting the quantification of microtubule anisotropy (n=18, progenitor cells (0.0292±0.0039) non-progenitor cells (0.05778±0.0046), ***P=5.879e-07, Paired t-test). **(E-G)** Representative images showing 30-celled productive progenitor (n=14) where the CLASP marked MTs in non-progenitor cells (yellow bordered) are anisotropic (red asterisk) (E, F) compared to the progenitor cells (green bordered) (E,G). (G) is the zoomed image from (E). Magenta arrows: aggregation of CLASP domains. **(F)** is the zoomed image from (E). GFP/YFP merged channel (E), YFP channel (F,G) YFP-CLASP, PIN1-GFP. **(H)** Schematic depicting the mechanical heterogeneity and MT orientation in a productive progenitor. **(I-L)** Representative time lapse image of the pseudo-progenitor (D1 of the time lapse in Figure 5.3.B), showing progenitor abortion marked by disappearance of PIN1-GFP by D5 (L). Scale bars: 50µm, n=sample number. Error bars represent s.e.m.

were analyzed to assess the orientation quantitatively, providing a clearer picture of the MT anisotropy differences. The quantitative data confirmed that surrounding non-progenitor cells indeed have significantly higher MT anisotropy than progenitor cells (Figure 5.3.D). Thus, the non-progenitor cells encapsulating the rapidly growing progenitor experience a circumferential stress field. This observation aligns with the previous studies which demonstrates that radial action of internal pressure acting on the boundary of a vessel, results in circumferential stress (Hamant *et al.*, 2008). Simply put, stress is circumferential in the cells surrounding a growing organ (Hervieux *et al.*, 2017).

The circumferential orientation of MTs in surrounding cells was, however, not present at earlier stages. In both the early (4- to 6-celled) productive progenitors and the pseudo-progenitors of comparable stages, this circumferential MT orientation was undetected, suggesting that it develops only after progenitors reach a threshold size and growth rate (Figure 5.3.A - 5.3.B). Pseudo-progenitors, which typically abort development at the 6- to 10-celled stage, never reached a stage that displayed distinct MT orientation differences between progenitor and non-progenitor cells (Figure 5.3.I - 5.3.L). This

observation further reinforces the idea that the MT alignment in non-progenitor cells depends on a specific growth context which is unique to productive progenitors.

In summary, I find that microtubules have isotropic orientation in the rapidly growing progenitor cells and anisotropic orientation in the surrounding non-progenitor cells, reflecting the circumferential mechanical stress experienced by the non-progenitor cells (Figure 5.3.H). This circumferential stress pattern develops in the surrounding non-progenitor cells only once progenitors reach a critical size and growth rate.

V.2.4. The progenitor cells are under compression and the surrounding non-progenitor cells are under tension

Reasoning that the progenitor and non-progenitor cells exist in distinct stress fields, I investigated the forces responsible for generating these stress fields. It is widely known that if the outer region of a sphere resists growth more than the inner region, the tissue behaves like a pressurized vessel, with the inner tissue experiencing compression and the outer tissue under tension (Hamant *et al.*, 2008). In that case, if one cell were removed from the compressed vessel, the gap left behind would be immediately closed. I found that ablating a single PIN1-GFP marked cell from 10- to 12-celled progenitors (n=6) prompted the adjacent PIN1-marked cells to quickly expand and close the gap within just 5 hours, indicating that the rapidly growing inner progenitor cells are indeed under compression (Figure 5.4.A - 5.4.D). Furthermore, ablating 3-4 non-progenitor cells (Figure 5.4.A - 5.4.P) caused adjacent progenitor cells to lose compression, as seen in their outward bulging and loss of compact packing (Figure 5.4.G, 5.4.J). This response indicates that the surrounding non-progenitor

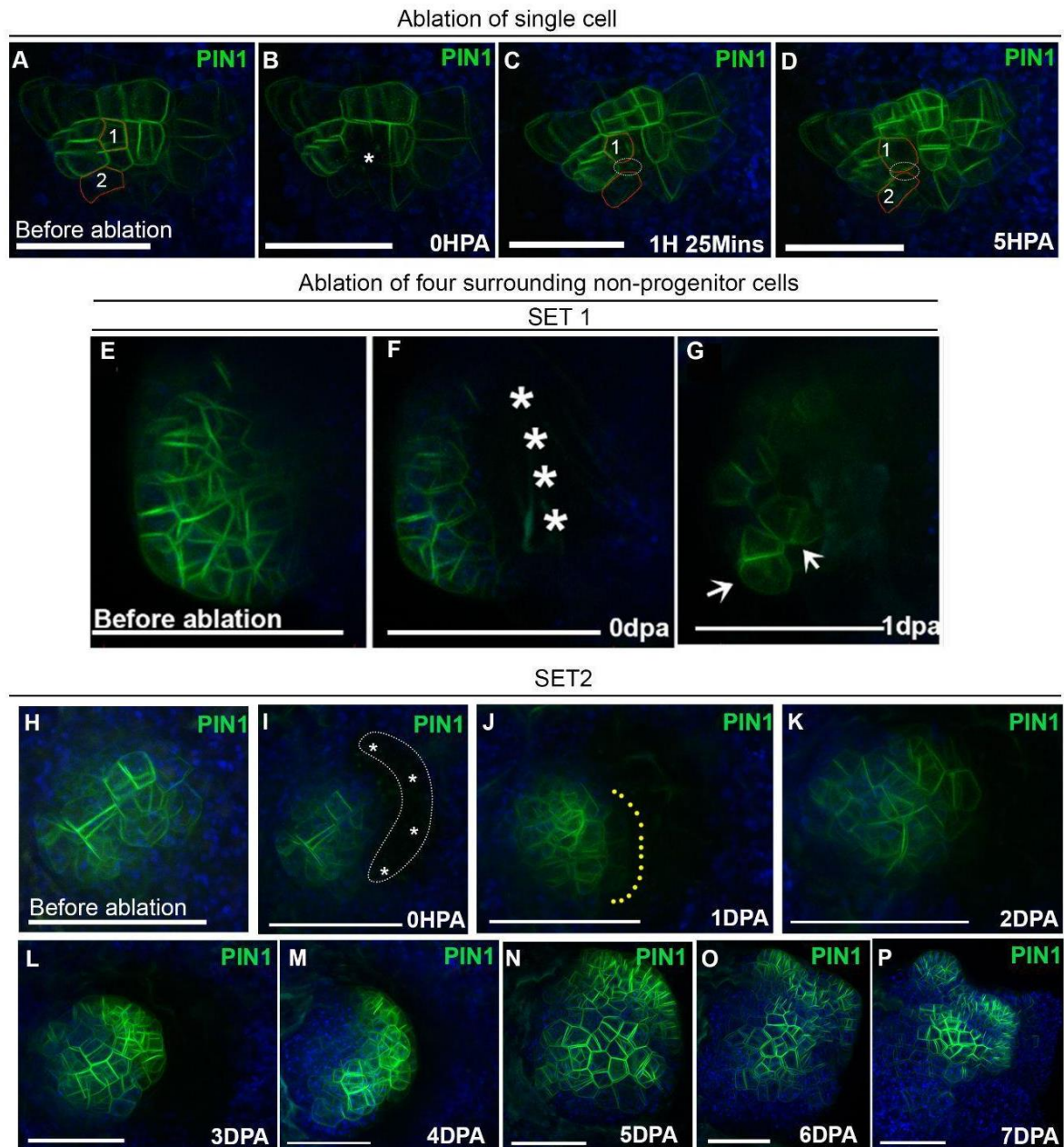


Figure. 5.4 : Progenitor cells are compressed by the shell of non-progenitor cells surrounding them during *de novo* shoot meristem formation (A-D) Representative time lapse images showing that ablation of a single PIN1- marked cell from the progenitor where cells are compressed into tight packing, triggered the adjoining PIN1 marked cells to expand into the gap generated and close the wound. White dotted circle between cells 1 and 2 shows the gap generated at 1Hr 25 mins post ablation(C). By 5hours post ablation, the gap got closed by the expansion of cells 1 and 2 (D). **(E-G)** Real-time live imaging of a productive progenitor following ablation. Ablation of four consecutive non-progenitor cells created a

lesion overarching the progenitor (F). White asterisk indicates the points of ablation. By 1-dpa, the progenitor cells showed a disoriented PIN1-GFP pattern, loss of compact arrangement, increase in curvature, and appeared to fall apart (G). **(H-P)** Representative time lapse images showing that targeted laser ablation of four consecutive non-progenitor cells surrounding a 15 celled progenitor generates a lesion of small arc length which impinges on the enclosed progenitor cells, such that it progressed into shoot meristem only after prolonged incubation on SIM (n=22). Note that the walls of the progenitor cells facing the lesion have increased in curvature. Yellow dots indicate the increase in curvature of the progenitor cells in response to ablation of its adjoining non-progenitor cells. Scale bars: 50µm, n=sample number.

cells are under tension, applying compressive forces on the progenitors. The immediate change in behavior of the direct neighbors following ablation aligns with previous studies showing that cell ablation affects the mechanical properties of adjacent cells (Hamant *et al.*, 2008; Hoermayer *et al.*, 2020; Long *et al.*, 2020).

Together, these results reveal a balance of two conflicting forces, where rapidly growing progenitor cells experience compressive force from the surrounding slower-growing non-progenitor cells that maintain tension. Perturbing this balance of forces impairs the PIN1 localization leading to progenitor abortion.

V.2.5. Meristem formation requires the coordinated interaction between progenitor cells and surrounding non-progenitor cells

Next, I investigated whether physically perturbing the balance between the two conflicting forces could impair progenitor progression. To do so, I used laser-targeted cell ablation to remove a few cells and disrupt the forces. I began with a control experiment to standardize the parameters for cell ablation, aiming to minimize the stress response caused by the ablation while ensuring that the consequences did not hinder recovery (Figure 5.5.A

- 5.5.E). For the control experiments, I ablated a single PIN1-GFP marked cell from the surface of 7- to 8-celled shoot progenitors (n=4) (Figure 5.5.B). By 1 day post-ablation (dpa), new PIN1-GFP cells replaced the ablated cells, and the progenitor progressed into a shoot meristem by 3 -dpa (Figure 5.5.E), indicating successful standardization of the ablation procedure. In the experimental setup, I initially perturbed the tension by ablating surrounding non-progenitor cells. Strikingly, creating a long lesion overarching a 15-celled progenitor by ablating 8-10 XTH9-marked non-progenitor cells disrupted progenitor progression entirely (n=34) (Figure 5.5.F - 5.5.I). Tracking the sequential abortion of the progenitor showed a reduction in PIN1-GFP expression by 1-dpa and a complete loss of PIN1-GFP by 2-dpa (Figure 5.5.H). However, a shorter lesion created by ablating 4 non-progenitor cells, as seen in the previous section, did not abort the 15-celled progenitor (n=22) (Figure 5.4.I). Instead, it delayed the progenitor's progression (Figure 5.4.H - 5.4.P). The delay was evident, as the progenitors took 7 days to develop into a meristem (5.4.P), whereas unablated progenitors at similar stages took less than 5 days (Figure 1.1.D - 1.1.F). Tracking progenitor progression during the delayed period revealed that PIN1-GFP diminished from the cell membrane and progenitor progression temporarily halted at 2-dpa (Figure 5.4.K). Only when robust PIN1-GFP expression was reinstated by 3-dpa did the progenitor resume progression, further highlighting the importance of proper PIN1 expression for progenitor progression (Figure 5.4.L - 5.4.P). Taken together, these results suggest that surrounding non-progenitor cells and the tension maintained by them are essential for maintaining proper PIN1 expression and enabling progenitor progression to the meristem.

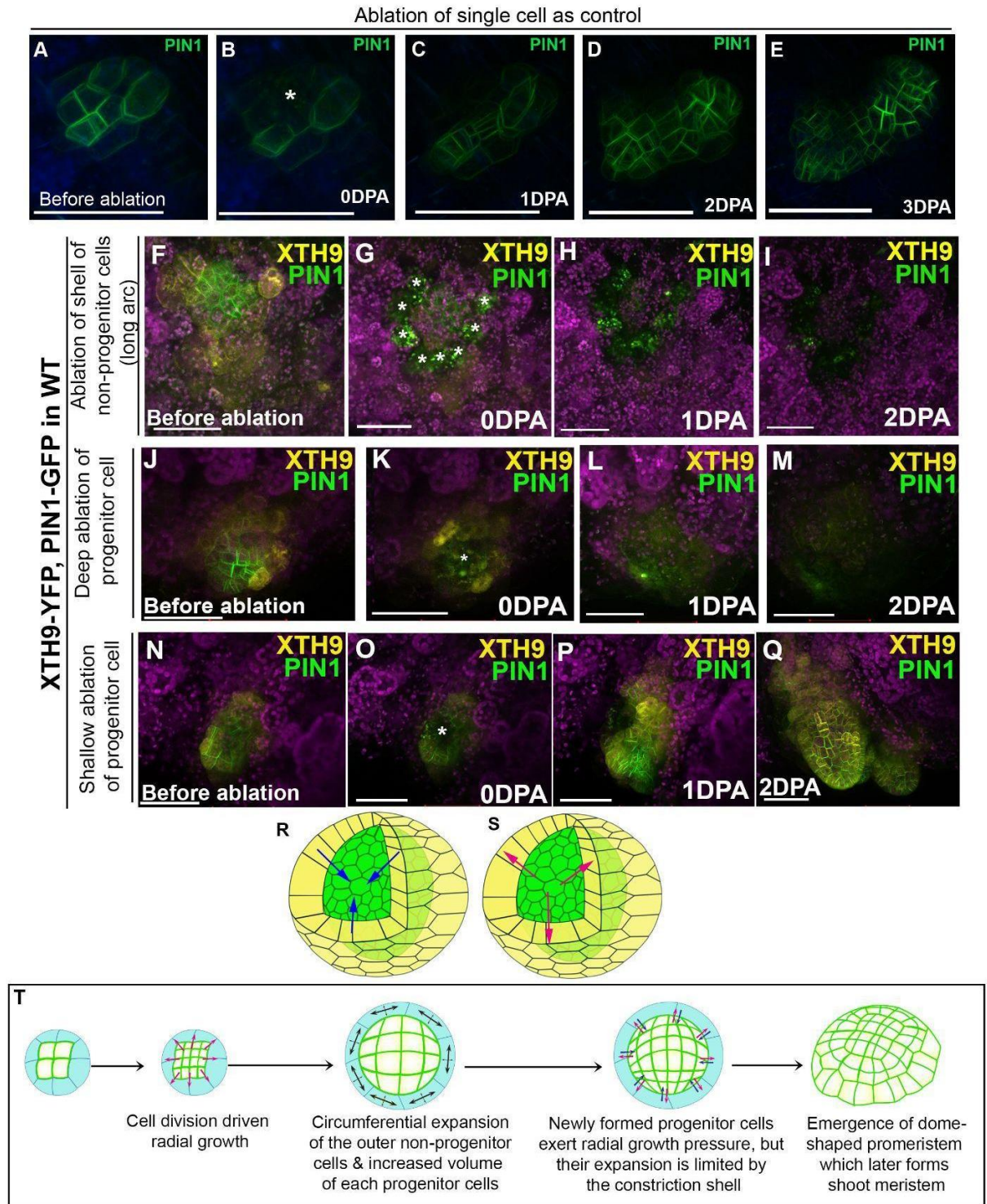


Figure. 5.5: *De novo* shoot meristem formation necessitates mechanical conflict between progenitor and its surrounding cells. (A-E) A control experiment where ablation

of the central cells of a progenitor (7 celled stage), did not impede its progression or function and made a functional shoot meristem by 3-day post ablation (dpa)(n=4). **(F-I)** Representative real-time live images following ablation of XTH9-marked non-progenitor cells. **(J-M)** Representative real-time live images following deep ablation (45 μm) of a progenitor. **(N-Q)** Representative real-time live images following shallow ablation (25 μm). **(R-S)** Schematic representation of the feedback between the progenitor and the surrounding shell of non-progenitor cells **(T)** Self-perpetuating hypothetical model, showing feedback between progenitor (pale yellow) and non-progenitor cells(blue). Red arrows: radial growth and outward pressure of progenitor cells (green). Black double-headed arrow: direction of anisotropic stress in non-progenitor cells. Dark-blue arrows: compression from the constriction shell. Scale bars: 50 μm , n:sample number.

Next, I directly ablated the PIN1-GFP marked progenitor cells to perturb the compressive forces. A narrow, single-cell-wide but deep ablation (45 μm depth) through the center of 10- to 15-celled progenitors led to their abortion by 2-dpa (n=39) (Figure 5.5.J - 5.5.M). Real-time tracking of events leading to abortion showed the disappearance of PIN1-GFP from the progenitor's cell membranes by 1-dpa, accompanied by a dramatic decrease in XTH9 expression in surrounding non-progenitor cells (Figure 5.5.L). By 2-dpa, the progenitor was fully aborted, marked by the complete loss of residual PIN1-GFP (Figure 5.5.M). The dramatic phenotype caused by deep ablation made it challenging to document the transient changes in cellular behavior. I, therefore, performed shallow (25 μm deep) ablations to mitigate the dramatic effects of deep ablations. A shallow ablation on 10- to 15-celled progenitors caused the PIN1-GFP cells at the periphery, left behind after the ablation, to form a new progenitor near the original position (Figure 5.5.N - 5.5.P). This new progenitor then developed into a shoot meristem (n = 25) (Figure 5.5.Q). I captured the transient changes in PIN1-GFP localization and XTH9 expression patterns that occurred between ablation and the establishment of the new progenitor. Time-lapse imaging over 3 days at 24-hour intervals revealed that the remnant cells at the periphery re-oriented their

PIN1-GFP localization pattern and self-organized into a new progenitor by 1-dpa. Concurrently, XTH9-YFP disappeared from around the ablated progenitor and simultaneously appeared around the newly formed progenitor, all by 1-dpa (Figure 5.5.P). While I do not discount the possibility of other intercellular signals communicating between progenitor and non-progenitor cells, the results suggest that the progenitor and non-progenitor cells work in coordination and likely depend on each other for proper progression.

The coordination between progenitor and surrounding non-progenitor cells was further validated through genetic means. Perturbing the non-progenitor cells, such as by genetically altering the CUC2-XTH9 regulatory axis, resulted in loss of PIN1 expression and localization and eventually the abortion of progenitors. *Vice versa*, impairing PIN1 expression in progenitor cells—either through genetic deregulation or loss of function—led to the downregulation of both CUC2 and XTH9 in the surrounding non-progenitor cells (Varapparambath and Mathew et al., 2022). Thus, disrupting the CUC2-XTH9 axis in non-progenitors impairs PIN1 localization in progenitors, and perturbing PIN1 localization disrupts the CUC2-XTH9 axis. This shows that physical or genetic perturbations to either the progenitor or non-progenitor cells feedback on each other, resulting in disrupted PIN1 localization in progenitors and preventing their progression to the meristem. This CUC2-XTH9 axis is crucial for shoot regeneration but dispensable for the formation of the pre-patterned shoot apical meristem during normal development (Varapparambath and Mathew et al., 2022). This is not surprising, as the embryonic SAM forms directly on the surface without emerging through overlaying cell layers, unlike the meristem that regenerates from progenitors. However, once the *de novo* meristem is established, it recapitulates the

expression patterns of the regulators tested in this study, similar to those observed in the developing SAM.

In summary, these findings show that the tension in non-progenitor cells and compression in progenitor cells rely on each other to maintain a balance of forces (Figure 5.5.R - 5.5.S). The feedback, whether mechanical or biochemical, between the progenitor and surrounding non-progenitor cells is essential for proper progenitor progression to the meristem during *de novo* shoot regeneration.

V.2.6. Making of the shoot meristem from shoot progenitors *de novo* is a self-sustained process

I propose a self-sustained 'rubber-shell' model, where the surrounding non-progenitor cells expressing XTH9 act like a rubber shell with both compressive and expansive properties (Figure 5.5.T). Based on my findings that progenitor cells undergo prolific division and are under compression, while surrounding non-progenitors, which divide rarely, are under tension (Figures 5.2, 5.4), I hypothesize the following: The rapid growth of inner progenitor cells generates radial pressure that, when exceeding the shell's compression, forces the rubber-shell-like non-progenitors to expand. This expansion accommodates the rapidly growing progenitor and encourages further division of progenitor cells, creating a positive feedback loop that drives both shell expansion and progenitor compression. This feedback loop likely continues until the progenitor develops into a meristem. The mechanical conflict at the boundary, where compressed progenitor cells meet tensioned non-progenitor

cells, likely causes the progenitor to buckle into a dome shape, which eventually breaks through the shell to form the meristem.

My observations suggest that the cell wall-loosening enzyme XTH9 facilitates the expansion of non-progenitor cells, allowing them to stretch without breaking and support progenitor growth. Consistent with this, in *xth9* mutants where the shell fails to expand, the progenitor does not grow and eventually aborts (Figure 4.3). This indicates that XTH9-driven expansion in non-progenitor cells is crucial for relieving mechanical constraints, supporting progenitor growth, and preventing arrest. This result echoes studies on the growth of Madin-Darby Canine Kidney (MDCK) cell colonies, where slow expansion of peripheral cells increases central cell density, triggering a proliferation arrest due to space limitations (Puliafito *et al.*, 2012). Similarly, the finding that progenitor progression to the meristem relies on mechanical conflict aligns with research in animal systems, where early mesoderm specification depends on mechanical forces (Brunet *et al.*, 2013).

In summary, the 'rubber-shell' model illustrates how XTH9-mediated expansion in tensed non-progenitor cells enables compressed progenitor cells to grow. This coordinated balance of compression and tension creates mechanical conflict at the boundary, causing the progenitor to buckle into a dome-shaped meristem.

V.3. DISCUSSION

In this chapter, I uncovered how the dynamic interplay between progenitor and surrounding non-progenitor cells orchestrates the progression of shoot progenitor into shoot meristem. My results show that progenitor cells grow and divide significantly faster than

their neighbors, creating distinct geometries and mechanical stress fields between the two cell populations. While progenitor cells expand isotropically, non-progenitor cells adopt an elongated, anisotropic shape, suggesting they are stretched by the fast-growing progenitors. This difference in growth rates establishes a balance between tension and compression, with progenitor cells under compression and non-progenitor cells under tension. Ablation experiments and genetic perturbations further reveal that maintaining this balance is crucial for cell polarity and for formation of shoot meristem *de novo*. Altogether, these findings led me to postulate a ‘rubber-shell’ model in which non-progenitor cells act as a flexible shell that expands around the proliferating progenitors, allowing for progenitor growth while generating a mechanical conflict at their shared boundary. This conflict, arising from opposing forces of compression in the progenitors and tension in the non-progenitors, drives the buckling of progenitor cells into a dome-shaped shoot meristem. In short, the mechanical conflict caused by cell wall loosening enzyme, XTH9 activates cell polarity and promotes progenitor progression during *de novo* shoot regeneration.

While this chapter reveals the critical role of mechanical feedback loops in *de novo* shoot meristem formation, it also underscores the broader significance of cellular interactions in driving self-organized morphogenesis. D’Arcy Thompson (1917) and others highlighted the role of mechanical forces in shaping biological structures, an idea that was later expanded upon by developmental biologists such as Bryant and Simpson (1984), who proposed that growth and patterning result from position-specific interactions between cells and their neighbors (Thomson, 1917; Bryant & Simpson, 1984). More recent studies across several biological systems confirm the indispensable role of physical forces in

morphogenesis (Nelson *et al.*, 2005; Aegerter-Wilmsen *et al.*, 2007; Egginton, 2011; Puliafito *et al.*, 2012; Uyttewaal *et al.*, 2012; LeGoff & Lecuit, 2015; Sampathkumar, 2020). In the developing SAM, for instance, the epidermis experiences tension, acting like an inflated shell that sustains internal pressure and influences growth in underlying tissues (Beauzamy *et al.*, 2015). This behavior mirrors the ‘rubber-shell’ model I propose, where non-progenitor cells form a flexible shell around progenitors, facilitating their growth while generating mechanical tension.

Despite these insights, several limitations remain. While the ‘rubber-shell’ model builds on observed mechanical interactions, a major limitation is the gap in understanding how cells sense and translate these forces. One of the strongest pieces of evidence supporting translation of mechanical signals into cellular responses comes from studies in Arabidopsis SAM, where STM expression has been shown to be induced by mechanical perturbations, suggesting a link between mechanical force (Landrein *et al.*, 2015). Similarly, in animals, the transcriptional co-activator YAP/TAZ—orthologous to Yorkie in *Drosophila*—has been implicated in translating mechanical signals to control growth and proliferation (Dupont *et al.*, 2011; Wada *et al.*, 2011). These findings support the idea that mechanical forces not only shape cellular architecture but also influence gene expression, driving developmental processes. While the ‘rubber-shell’ model provides a foundational framework for understanding progenitor and non-progenitor cell interactions, further studies should explore the complexity of these interactions. Investigating factors such as turgor pressure, cell wall properties, and additional cellular processes could further refine our understanding of these interactions. Expanding the study to include non-shoot-forming callus cells would also

enhance insight into how mechanical principles function across the entire callus. Additionally, comparing *de novo* shoot meristems with developing SAM could reveal conserved mechanisms, especially at organ boundaries where differential growth necessitates mechanical compromises (Hamant & Traas, 2010). Such research has potential applications for engineered plant systems and crop improvement, providing new perspectives on plant growth and regeneration.

***Chapter VI: Mapping the pattern
of mechanical heterogeneity
promoting the development of
progenitor during de novo shoot
regeneration***

Part of this work is in collaboration with:

Prof.Joris Sprakel, Maarten Besten and Prof.Dolf Weijers, WUR, Netherlands (Mathew

M.M et al., *unpublished*)

VI.1 INTRODUCTION

"In the midst of chaos, there is also opportunity."
— Sun Tzu, *The Art of War*

The mechanical behavior of biological tissues has been an active area of research for decades due to its critical role in regulating tissue homeostasis. Mechanical models of pattern formation have advanced our understanding of developmental processes across biological kingdoms, illustrating how cells physically interact with their surroundings (Villa *et al.*, 2021). The studies implicate that cells respond to mechanical forces—such as stretch, compression, tension, and shear—by altering their membrane structures, converting these forces into chemical signals, and ultimately modifying their behavior and fate (Ayad *et al.*, 2019). Importantly, cells or tissues with different mechanical properties respond differently to the same force, highlighting the need to study mechanical properties and their spatial patterns. There is mounting evidence showing that biological systems exhibit spatiotemporally patterned mechanical properties, which significantly influence growth, morphogenesis, development, and regeneration (Trubuil *et al.*, 2021). For example, during *Drosophila* gastrulation, cortical tension-driven mechanical patterns are essential for mesoderm invagination and tissue deformation (Rauzi *et al.*, 2015). Similarly, epithelial wound healing in *Drosophila* relies on mechanical heterogeneity, where softer cells near the wound facilitate tissue deformation for wound closure (Brugués *et al.*, 2014). Furthermore,

the modulation of these mechanical patterns can impact growth and development. For instance, during optic tract development in *Xenopus laevis*, axonal growth is guided by dynamic stiffness gradients in adjacent brain tissue (Koser *et al.*, 2016).

The growing recognition of mechanosensitivity—the ability of cells to respond to mechanical signals—has made accurate quantification of mechanical properties and their distribution patterns a critical area of study. Key properties that influence cellular and tissue mechanics include forces, cell geometry, and material properties, all of which exhibit dynamic patterning (Schlüßler *et al.*, 2018; Maroudas-Sacks & Keren, 2021). Over the years, several techniques have been developed to map and quantify these properties and their spatial distributions. Selecting an appropriate technique depends on the biological sample's scale and the resolution required for analysis. At the tissue scale, traditional *in vitro* methods such as compressive testing (Galford & McElhaney, 1970) and indentation (Budday *et al.*, 2015) have been widely used. Higher-resolution measurements at tissue and cellular levels often rely on Atomic Force Microscopy (AFM), which has become the gold standard in mechanobiology despite its limitations, such as sample deformation during measurements (Allison *et al.*, 2010). Other approaches include magnetic bead twisting rheology (Massiera *et al.*, 2007), optical tweezers (Nijenhuis *et al.*, 2009), and micropipette aspiration (Sato *et al.*, 1990). Laser ablation offers a complementary technique by measuring intracellular tension through the retraction of structures following targeted cutting with high-intensity laser beams (Ayad *et al.*, 2019). However, these methods share significant limitations, such as perturbing internal stresses with external forces, destructively accessing tissues, or providing insufficient spatial resolution.

To address these challenges, advanced microscopy-based techniques have emerged. Fluorescence resonance energy transfer (FRET) enables the measurement of mechanical tension at the molecular level (Grashoff *et al.*, 2010). Brillouin microscopy offers a non-destructive, label-free, and contact-free method to probe the material properties of biological samples in three dimensions (Riquelme-Guzmán *et al.*, 2022). Additionally, rigidochromic dyes allow for the study of microviscosity in cells and membranes (Kuimova *et al.*, 2008). These innovative approaches provide valuable insights while minimizing sample disturbance, significantly advancing the field of mechanobiology.

In plants, turgor pressure (TP), along with the structure and composition of the plasma membrane and the stiff yet malleable cell wall, regulates numerous biological processes. However, mapping the distribution patterns of TP and the mechanical properties of the plasma membrane and cell walls across scales—from subcellular structures to multicellular tissues—remains a challenge. Among the mechanical properties, the microviscosity of subcellular structures and spaces is particularly important. Microviscosity influences the diffusion rates of cellular molecules and organelles, impacting metabolism (Puchkov, 2013) as well as processes like signaling and enzymatic reactions (Gavish & Werber, 1979; Uribe & Sampedro, 2003). Techniques such as AFM, Brillouin microscopy, fluorescence recovery after photobleaching (FRAP), and particle tracking microrheology have been used to measure the viscosity in living plant cells and stiffness in plant cell walls (Carisey *et al.*, 2011; Milani *et al.*, 2013; Elsayad *et al.*, 2016; Long *et al.*, 2020). However, these methods still lack the ability to map and measure properties with sufficient resolution in three dimensions. To override this limitation, plant-specific rigidochromic dyes have been

developed, utilizing phenyl-substituted boron dipyrromethane (ph-BODIPY) molecular rotors. Upon photoexcitation, these rotors undergo intramolecular rotation, with the rate of rotation determined by the surrounding environment. The fluorescence lifetime and intensity of the BODIPY probes are directly influenced by rotational constraints, providing a means to measure the microviscosity.

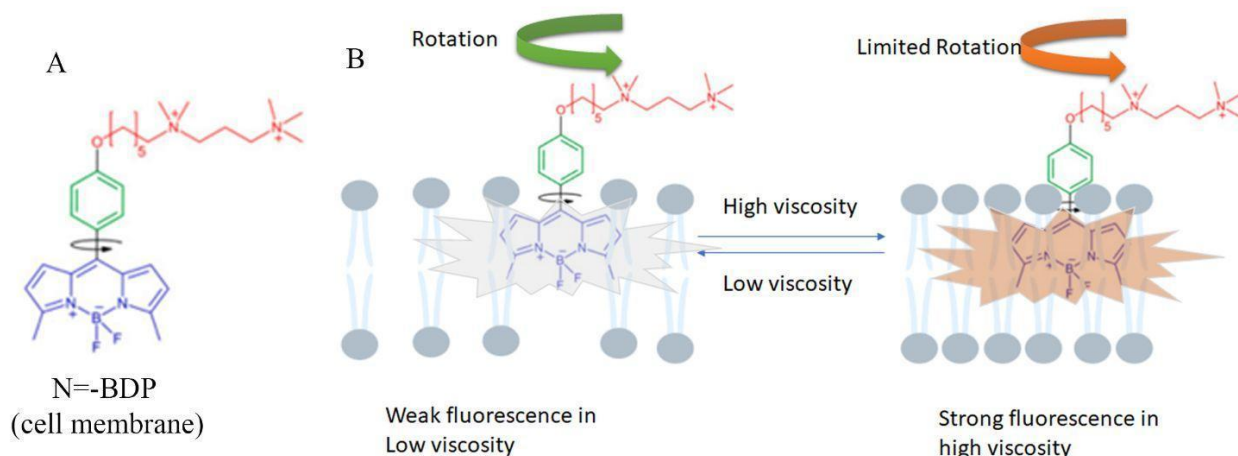


Figure. 6.1: Schematic representation of the N⁺BDP rigidochromic plasma membrane probe and its working principle.

Low fluorescence lifetime corresponds to low viscosity, where the probe rotates freely, while high fluorescence lifetime reflects hindered rotation and higher viscosity (Michels *et al.*, 2020; Besten *et al.*, 2024) (Figure 6.1).

To enable targeted measurements, substitutions on the phenyl ring allow specific localization of the BODIPY probe to various plant organelles. For example, a phenyl ring modified with an aliphatic tail carrying two permanent cationic charges (N⁺ BDP) targets the plasma membrane by getting inserted between negatively charged phospholipids (Michels *et al.*, 2020). For the cell wall, BODIPY is coupled to the CarboTag (CT) motif, a small molecular motif that directs the probe to the cell wall, and the probe is called CarboTag-BDP (CT-BDP) (Besten *et al.*, 2024). These innovations provide precise tools to probe the mechanical properties of plant structures with high specificity and resolution.

In this study, I use *de novo* shoot regeneration in *Arabidopsis thaliana* as a model system to investigate the patterns of mechanical heterogeneity and construct comprehensive microviscosity maps of shoot progenitor, the surrounding shell of cells, and the rest of the callus. This represents the first instance of generating complete microviscosity maps in tissue-cultured samples across biological kingdoms. Furthermore, I compare the microviscosity patterns in wild-type shoot progenitors to those observed under perturbations of mechanical properties, induced either physically or genetically. By integrating these analyses, this study sheds light on the spatial and quantitative distribution of mechanical properties and their role in regulating development and regeneration. This chapter not only offers novel insights into the mechanobiology of plant tissues but also establishes a methodological foundation for studying mechanical heterogeneity in other biological systems. These findings have far-reaching implications for understanding how mechanical cues govern cellular behavior, tissue morphogenesis, and regenerative processes across organisms, paving the way for future interdisciplinary explorations.

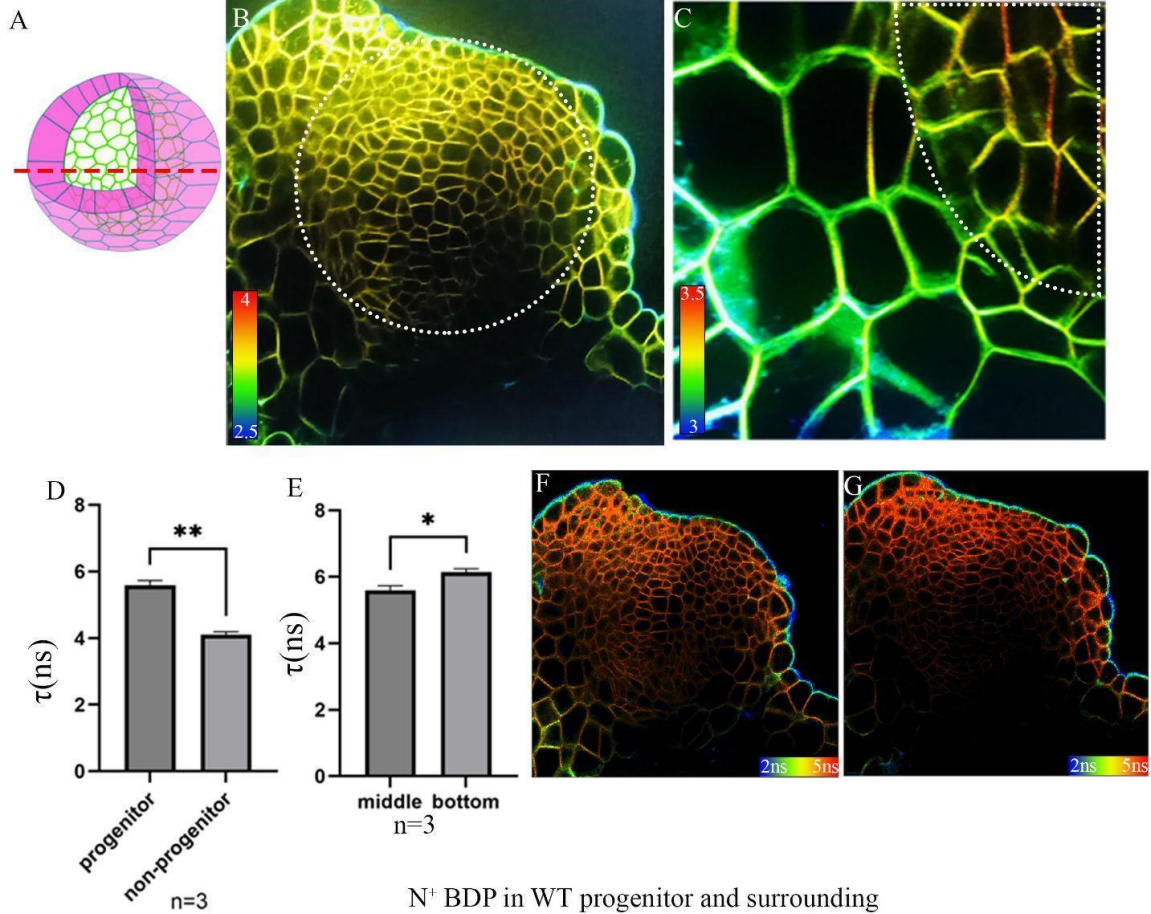
VI.2. RESULTS

VI.2.1. The callus cells are mechanically heterogeneous: progenitor cells are the most compressed

In Chapter 5, I established that the callus is mechanically heterogeneous, with shoot progenitor cells and the surrounding shell of non-progenitor cells exhibiting distinct forces and mechanical properties, and that this mechanical heterogeneity is critical for *de novo* shoot regeneration. Building on this observation, I examined the pattern of mechanical properties within a regenerating SIM callus and quantified it. Specifically, I generated a comprehensive microviscosity map of the SIM callus, encompassing both progenitor and non-progenitor cells, using the plant-specific Ph-BODIPY rigidochromic probe (Figure 6.1, Figure 6.2).

The mapping and quantification of the probe's rotational dynamics were achieved through confocal-based Fluorescence Lifetime Imaging Microscopy (FLIM). For this analysis, I excised 0.5 cm sections from 7–10-day-old SIM callus and placed them in 35 mm Petri dishes supplemented with solid SIM medium (as detailed in Chapter 2). Samples were stained with 10 μM of the plasma membrane rigidochromic probe, N⁺BDP, for 4 hours, washed with liquid SIM, and subjected to FLIM. Live imaging revealed that N⁺BDP in the progenitor cells exhibited high fluorescence lifetime distributions, with a lifetime of 5.6 ns, indicating higher microviscosity and compression. In contrast, the surrounding shell of non-progenitor cells showed a lower lifetime of 4.11 ns, reflecting reduced microviscosity

N⁺ BDP in WT progenitor and surrounding non-progenitor cells (Middle layer)



N⁺ BDP in WT progenitor and surrounding non-progenitor cells (Bottom layer)

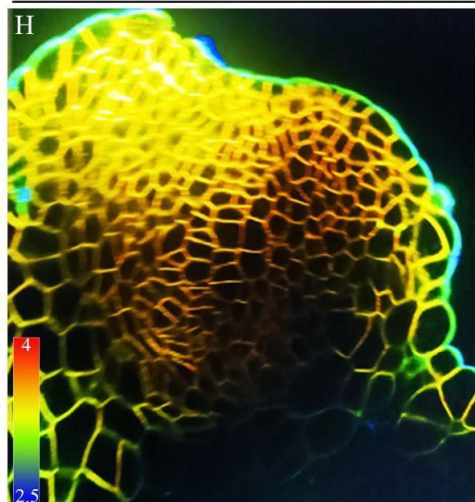


Figure. 6.2: The progenitor cells have higher membrane microviscosity than their surrounding non-progenitor cells. (A) Schematic representation of a middle plane of the progenitor (n=3). (B) Representative FLIM-based live confocal image of a progenitor stained with N⁺BDP though its middle section showing the heterogeneity in the microviscosities of progenitor and surrounding non-progenitor cells. (C) Zoomed in FLIM-based live confocal image of the progenitor from B. White dotted line encloses the progenitor. Scale represents the range of fluorescence intensity from FAST FLIM. (D) Graphical representation of the fluorescence intensity of the N⁺BDP in the progenitor and surrounding non-progenitor at the middle plane (Mann Whitney U-test; **p-value = 0.0079) (E) Graphical representation of the fluorescence intensity of the N⁺BDP in the progenitor at the middle plane and plane from the bottom of the progenitor (Mann Whitney U-test; *p-value = 0.0173). (F-G) Representative FLIM based confocal images (fitted images) showing the fluorescence lifetime of the N⁺BDP probe at the middle plane (F) and at a plane at the bottom of the progenitor (G). Scale: The fluorescence lifetime of the probe in nanoseconds (ns). (H) Representative FLIM-based live confocal image of a progenitor stained with N⁺BDP though plane from the bottom of the progenitor, showing the heterogeneity in the microviscosities of progenitor and surrounding non-progenitor cells. Scale represents the range of fluorescence intensity from FAST FLIM. Error bars: S.E.M. n= sample number.

(Figure 6.2.A - 6.2.D). I further mapped and measured the microviscosity along the z-axis of the progenitor cells to examine how compression varied within the tissue. The results revealed that the degree of compression increased with depth. The fluorescence lifetime of the N⁺BDP plasma membrane probe rose from 5.6 ns at the middle plane of the progenitor to 6.14 ns at the bottom plane, indicating higher microviscosity, and therefore higher compression in the deeper tissue layers (Figure 6.2.E - 6.2.H). These observations revealed mechanical heterogeneity not only between progenitor and non-progenitor cells but also along the z-axis within the progenitor itself.

Using the probe's exceptional cellular and subcellular resolution, I examined the microviscosity patterns of the plasma membrane in individual progenitor cells. The analysis revealed that each side of a progenitor cell was mechanically distinct, with N⁺BDP lifetime values ranging from 5.3 ns to 5.72 ns (Figure 6.3.A - 6.3.C). In contrast, the non-shoot-

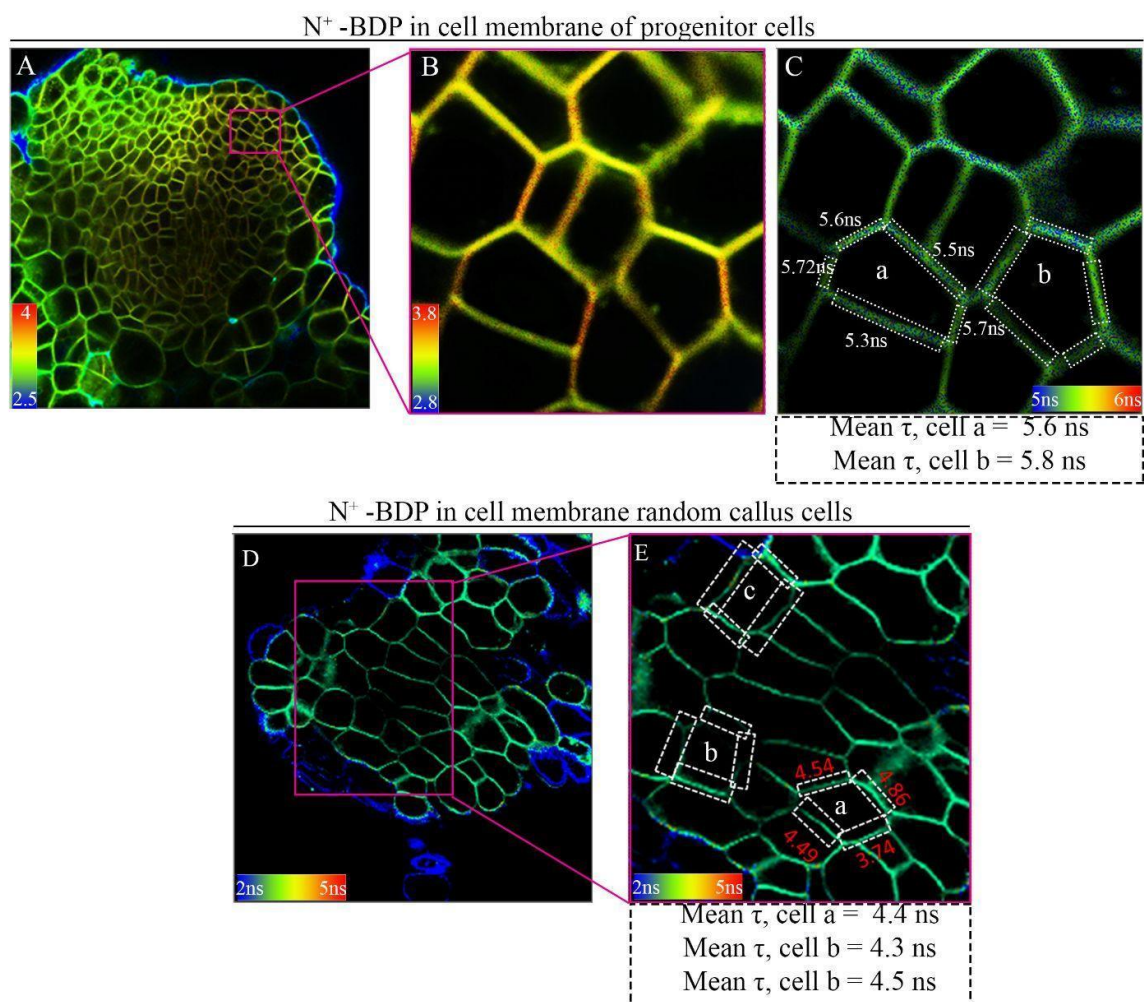


Figure. 6.3: The cells of the progenitor are mechanically heterogeneous even at subcellular scale. (A-B) Representative FLIM-based live confocal image of a progenitor stained with N^+ BDP though its middle section (A) with its progenitor cells showing the heterogeneity in the microviscosities at subcellular resolution (B; inset). Scale represents the range of fluorescence intensity from FAST FLIM. **(C)** Representative FLIM based confocal images (fitted images) showing the fluorescence lifetime of the N^+ BDP probe in different sides of progenitor cells. Scale: The fluorescence lifetime of the probe in nanoseconds (ns). **(D-E)** Representative FLIM based confocal image (fitted images) showing the fluorescence lifetime of the N^+ BDP probe in random non-shoot forming callus cells. E is the inset from D. Scale: The fluorescence lifetime of the probe in nanoseconds (ns).

forming callus cells, which constitute the bulk of the callus, exhibited less viscous plasma membranes. The N⁺BDP probe in these random cells had an average lifetime of 4.4 ns. Despite their lower viscosity, these cells also displayed mechanical heterogeneity at the subcellular level, with lifetime values for N⁺BDP ranging from 3.7 ns to 4.8 ns across different sides of the same cell (Figure 6.3.D - 6.3.E).

In summary, my quantifications demonstrated that the regenerating callus and developing shoot progenitors exhibit a distinct pattern of mechanical heterogeneity at the tissue, cellular, and subcellular levels.

VI.2.2. Removal of cell wall loosening activity from the surrounding shell of cells or hyperosmotic shock makes the shoot progenitor highly compressed during *de novo* shoot regeneration

I had previously shown in chapter 5, that removing the activity of the cell wall-loosening enzyme from the non-progenitor shell of cells surrounding the progenitor retarded progenitor growth and prevented its progression into a shoot meristem and a complete shoot system. I suggested that this was due to the inability of the surrounding shell to expand, leading to sustained compression of the enclosed progenitor cells. To test this, I examined whether the progenitor cells were excessively compressed and quantified the degree of compression by mapping the plasma membrane microviscosity in progenitors derived from the *xth9* mutant callus. The analysis revealed that the N⁺BDP probe lifetime in *xth9* mutant progenitors was significantly higher, with an average lifetime of 6.8 ns compared to 5.6 ns in wild-type progenitors, indicating increased viscosity in the mutant (Figure 6.4.A - 6.4.D).

Additionally, the non-progenitor shell of cells in the *xth9* mutant also exhibited a highly viscous plasma membrane, with an N⁺BDP lifetime of 6.25 ns compared to 4.11 ns in the wildtype (Figure 6.4.B). These results demonstrate that the absence of cell wall-loosening activity in the surrounding shell of cells renders the progenitor cells highly compressed.

I further investigated the pattern of microviscosity in the cell walls of WILDTYPE and *xth9* mutant-derived SIM callus. To achieve this, I stained the biological samples with 10 μ M CarboTag-BDP (CT-BDP) for 6 hours. CT-BDP is a rigidochromic dye designed to probe the porosity of cell walls. The qualitative observation showed that the fluorescence lifetime and intensity of the CT-BDP in *xth9* mutant callus cells were higher than those in wildtype callus cells (Figure 6.4.E - 6.4.F). This result indicated that the cell walls of *xth9* mutant calli were stiffer and less porous compared to wildtype calli.

In plant cells, two key mechanical properties that regulate growth and development are the cell wall and turgor pressure (TP). Having already shown that stiffening the cell wall of the surrounding cells compresses the progenitor, I investigated whether altering TP could similarly affect the mechanical properties of progenitor cells. To test this, I treated wildtype callus with 0.4 M and 0.8 M mannitol for 6 hours to induce hyperosmotic stress. The treated samples were stained with 10 μ M N⁺BDP, supplemented with corresponding mannitol concentrations, and imaged using FLIM. The analysis revealed that hyperosmotic stress qualitatively increased the microviscosity of the cell membrane.

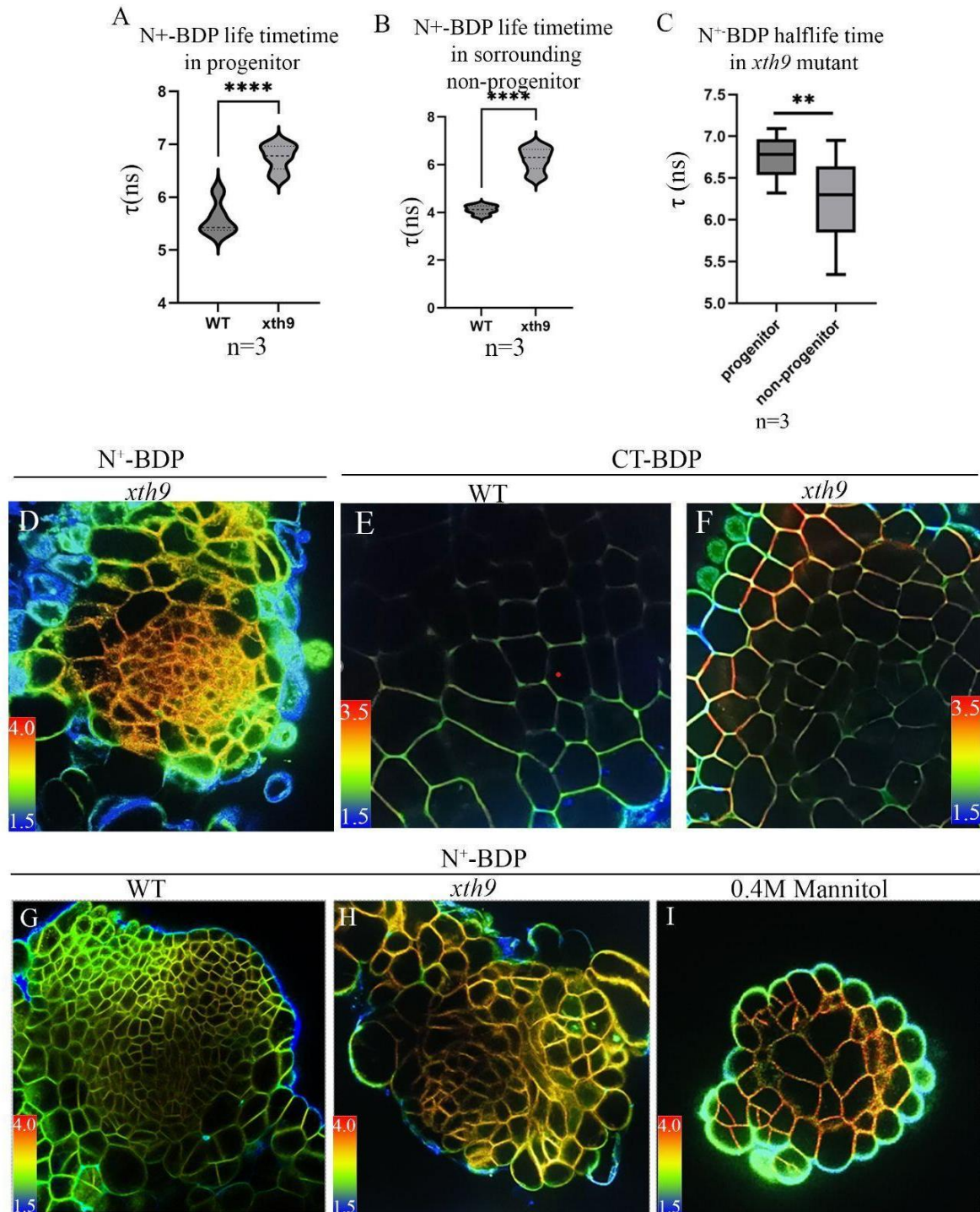


Figure. 6.4: The cell membrane microviscosity of progenitors increases in *xth9* mutant and upon hyperosmotic stress. (A) Graphical representations of N⁺BDP lifetime in wildtype v/s *xth9* mutant progenitor (Mann Whitney U-test; ****p-value < 0.0001 ; ROIs

analyzed in wildtype=5, ROIs analysed in *xth9* mutant=20). **(B)** Graphical representations of N⁺BDP lifetime in the surrounding non-progenitor cells of WILDTYPE v/s *xth9* mutant progenitor (Mann Whitney U-test; ****p-value < 0.0001 ; ROIs analyzed in WILDTYPE=5, ROIs analysed in *xth9* mutant=20). **(C)** Graphical representations of N⁺BDP lifetime in the progenitor and surrounding non-progenitor cells of *xth9* mutant progenitor (Unpaired t-test; **p-value = 0.0022 ; ROIs analyzed in wildtype=20, ROIs analysed in *xth9* mutant= 39). **(D)** Representative FLIM-based live confocal image of an *xth9* mutant progenitor stained with N⁺BDP through its middle section. **(E-F)** Representative FLIM-based live confocal image of callus cells in wildtype (E) and *xth9* mutant (F) stained with the cell wall porosity probe, CT-BDP. **(G-I)** Representative FLIM-based live confocal image of progenitor in wildtype (G) and *xth9* mutant (H) and after 0.4M mannitol treatment in wildtype (I) stained with N⁺BDP probe. Scale represents the range of fluorescence intensity from FAST FLIM. Error bars: S.E.M. n= sample number.

These results demonstrated that modulating TP, or altering cell wall properties, impacts the mechanical state of progenitor cells. Specifically, both *xth9* removal and hyperosmotic stress caused the progenitor cells to become highly compressed (Figure 6.4.G - 6.4.I).

VI.2.3. De novo shoot regeneration is hypersensitive to perturbations in mechanical properties

Next, I investigated whether changes in the mechanical properties of progenitor cells influenced their fate. In Chapter 5, I demonstrated that the *xth9* loss-of-function mutant caused delocalization of PIN1-GFP from the plasma membrane, which disrupted progenitor progression and ultimately abolished shoot regeneration. Since osmotic stress affects progenitor mechanics, I explored its impact on progenitor fate using live imaging. To test this, I treated *pSOK5:gSOK5-YFP*-expressing wildtype progenitors with 0.8 M mannitol for 8 hours. I observed that SOK5, an intrinsic cell polarity protein associated with PIN1-marked

cells of productive progenitors (as described in Chapter 3), disappeared immediately after treatment (Figure 6.5.A - 6.5.B). These progenitors failed to progress into shoot meristems or form functional shoots, demonstrating that *de novo* shoot regeneration is highly sensitive to osmotic stress.

To further explore the role of mechanical properties in *de novo* shoot regeneration, I employed a genetic approach, focusing on mutants with defects in cell wall properties: *feronia-4* and *xxt1,xxt2*. FERONIA (FER) is a pleiotropic pectin-binding Receptor-Like Kinase (RLK) critical for maintaining cell wall integrity (CWI) (Feng *et al.*, 2018). Loss of FER function has been reported to compromise CWI, resulting in accelerated growth and cell bursting during normal development (Feng *et al.*, 2018). Additionally, FLIM-based porosity measurements of cell walls in the *fer-2* and *fer-4* single mutants stained with CT-BDP revealed significantly increased wall porosity in roots (Besten *et al.*, 2024). In contrast, XYLOGLUCAN XYLOSYLTRANSFERASE (XXT) is an enzyme involved in the biosynthesis of xyloglucan, a major polysaccharide of the plant cell wall. It has been shown that the tensile strength of the cell walls in the *xxt1,xxt2* double mutant is significantly reduced compared to the wildtype during normal development (Sowinski *et al.*, 2022). My analysis revealed that *de novo* shoot regeneration was dramatically impaired in both *fer-4* single mutants and *xxt1,xxt2* double mutants (Figure 6.5.C - 6.5.E). Interestingly, despite the drastic reduction in regenerated shoots, the SIM callus in the *fer-4* mutant appeared significantly larger and more robust, with excess growth and proliferation compared to the wildtype. This finding suggests that the shoot regeneration defect in the *fer-4* mutant was independent of callus formation.

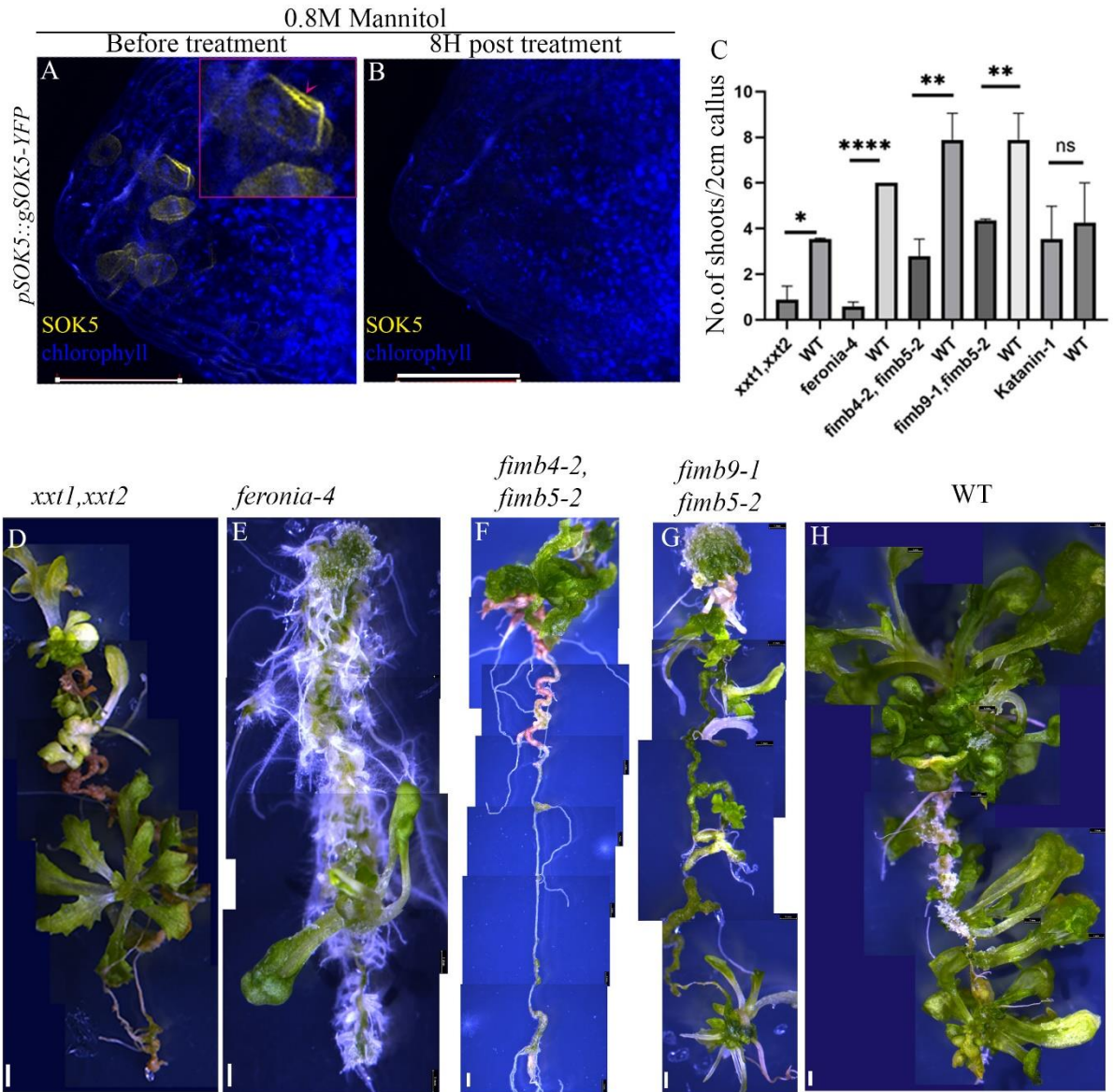


Figure. 6.5: De novo shoot regeneration is hypersensitive to cell mechanics. (A-B) SOK5-YFP disappears from the progenitor upon treatment with 0.8Mannitol for 8 hours. (C) Graphical representation of shoot regeneration frequency in loss-of-function mutants of *xxt1, xxt2* double mutant (n=120), *feronia-4* (n=150), *fimbrin4-2, fimbrin5-2* double mutant (n=200), *fimbrin9-1, fimbrin5-2* double mutant (n=30), and *katanin-1* (n=110) compared to wildtype (Unpaired t-test; *p-value=0.0418 between *xxt1, xxt2* double mutant and wildtype, ****p-value < 0.0001 between *feronia-4* mutant and wildtype; **p-value=0.0048 between *fimbrin4-2, fimbrin5-2* double mutant and wildtype; **p-value=0.0052 between *fimbrin9-1, fimbrin5-2* double mutant and wildtype; ns p-value=0.7711 between *katanin-1* mutant and

wildtype). **(D-H)** Representative stereo images of calli on 30th Day of SIM incubation. Scale bars: 50µm (A-B), 1mm (D-H). Error bars: S.E.M. n= sample number.

Next, to understand the impact of cytoskeletal mechanics on *de novo* shoot regeneration, I examined mutants with defects in cytoskeletal function: *katanin-1* and *fimbrin* loss-of-function mutants. KATANIN (KTN) is a microtubule-severing enzyme critical for cortical microtubule (CMT) organization and anisotropic cell expansion (Uyttewaal *et al.*, 2012), and FIMBRIN is an actin crosslinker whose detachment from actin filaments is thought to be mechanosensitive (Mousavi *et al.*, 2022). I found that *de novo* shoot regeneration in the *ktn-1* single mutant was unaffected and comparable to that of the wildtype. However, *de novo* shoot regeneration was significantly reduced in the *fimb4-2*, *fimb5-2*, and *fimb9-1 fimb5-2* double mutants (these mutants were originally screened and identified by Prof. Joris Sprakel's group at WUR, Netherlands, and are yet to be published) compared to the wildtype (Figure 6.5.C, 6.5.F-H). Notably, in all these mutants, normal shoot formation during development was unperturbed and comparable to that of the wildtype. This finding suggests that the mechanisms governing *de novo* shoot regeneration are distinct from those involved in normal shoot development during embryogenesis.

In summary, I find that *de novo* shoot regeneration is hypersensitive to modulation of mechanical properties, whether through alteration of turgor pressure (TP), cell wall mechanics, or cytoskeletal mechanics. These findings underscore the crucial role of mechanical cues in regulating developmental processes, where even subtle changes in cellular mechanics can dramatically impact the regenerative potential of progenitor cells.

VI.3. DISCUSSION

In this chapter, I examined the pattern of mechanical properties of progenitor cells during *de novo* shoot regeneration in *Arabidopsis thaliana*. Using advanced fluorescence lifetime imaging microscopy (FLIM) and plant-specific probes, I generated comprehensive microviscosity maps of regenerating callus tissue. These maps revealed the pattern of mechanical heterogeneity within the callus, particularly between progenitor and non-progenitor cells, as well as along the z-axis of the progenitor cells themselves. Furthermore, manipulation of the mechanical environment—through altering turgor pressure (TP), cell wall properties, or cytoskeletal mechanics—demonstrated that changes in mechanical properties profoundly impacted the regenerative capacity of progenitor cells. These findings highlight the importance of mechanical properties of cells and tissues, and their distribution pattern in controlling plant development and tissue regeneration. The role of fimbrin, an actin crosslinker, is particularly notable in this context. The mechano-sensitivity of fimbrin (Mousavi *et al.*, 2022) suggests that it could modulate actin dynamics in response to mechanical cues, thus impacting cell fate decisions. When fimbrin is impaired, as seen in the *fimbrin* loss-of-function mutants, shoot regeneration is compromised. This is likely due to disrupted mechanical signaling that alters the actin cytoskeleton, which in turn could affect how progenitor cells sense and respond to mechanical forces during regeneration. Microviscosity also influences cellular metabolism, as it regulates the diffusion rates of molecules and organelles within cells (Puchkov *et al.*, 2013). By affecting the intracellular movement and availability of key metabolic components, changes in microviscosity could directly impact cellular processes such as signaling and enzymatic reactions (Uribe *et al.*,

2003), and possibly the cell fate. Therefore, the pattern of mechanical properties of cells and tissues, including microviscosity, are integral not only for ensuring the proper functions necessary for regeneration, but I speculate also for defining cell fate.

Looking ahead, the findings of this study open several exciting avenues for future research. Further investigation into the molecular mechanisms that connect mechanical properties with gene expression and cellular behavior will be crucial in understanding how mechanical signals are transduced within plant cells. Additionally, exploring the role of mechanical feedback loops in other regenerative contexts—such as in roots, leaves, or even engineered plant tissues—could offer broader implications for agricultural practices and biotechnology. By advancing our understanding of how mechanical cues influence plant growth and regeneration, we may be able to devise novel strategies for enhancing crop yield, improving tissue culture protocols, and developing sustainable agricultural solutions in the face of environmental challenges.

***Chapter VII: Generation of cell
geometries guiding the shape of
regenerating organ***

Mathew M.M et al., *unpublished*

VII. 1. INTRODUCTION

"Form follows function—that has been misunderstood. Form and function should be one, joined in a spiritual union."

— Frank Lloyd Wright

The diversity of life on Earth stems from the ability of organisms to form distinct structures and shapes through complex developmental processes. In multicellular organisms, morphogenesis—the process by which tissues and organs acquire their shape—depends on coordinated cellular and tissue-level deformations (Coen *et al.*, 2004; Nelson, 2009; Coen & Rebocho, 2016; Cobham & Mirth, 2020; Hamant & Saunders, 2020; Kelly-Bellow *et al.*, 2023; Coen & Cosgrove, 2023). Studies in both plants and animals have highlighted shared mechanisms, such as bending, buckling, twisting, and turning, which contribute to shaping functional forms (Nath *et al.*, 2003; Coen & Rebocho, 2016; Hong *et al.*, 2017; Whitewoods & Coen, 2017; Huang *et al.*, 2018; Zhao *et al.*, 2020; Roeder, 2021; Varapparambath *et al.*, 2022; Wilson-Sánchez *et al.*, 2022; Satterlee *et al.*, 2023). In animals, these deformations are further complemented by cell migration, slipping, and folding, enabling the generation of intricate structures (Keller, 2002; Solnica-Krezel, 2005; Cobham & Mirth, 2020). Plants, however, face unique challenges due to their rigid cell walls. Instead of cellular migration, plant morphogenesis relies on changes in cell size, shape, division rates, and division orientation to achieve tissue-wide deformation. For example, in *Arabidopsis* sepals, alterations in growth rates and cell shapes can lead to significant changes in organ morphology (Hong *et al.*, 2016).

Roots are a critical plant organ, and their tapering shape and conical tips play a crucial role in regulating the root's elongation rate, and soil penetrance (Adorada *et al.*, 2000; Colombi *et al.*, 2017; Potocka & Szymanowska-Pułka, 2018). The development of root structure and patterning has been extensively studied, with computational models offering insights into the formation of the root's anisotropic shape during embryogenesis (Marconi *et al.*, 2021), as well as the auxin-dependent growth and zonation in post-embryonic roots (Grieneisen *et al.*, 2007; Mironova *et al.*, 2010; Band *et al.*, 2014; De Vos *et al.*, 2014; Mähönen *et al.*, 2014; Jensen & Fozard, 2015; Morales-Tapia & Cruz-Ramírez, 2016; Rutten & Ten Tusscher, 2019). Recent studies also suggested that Brassinosteroid signaling regulates both longitudinal and radial growth in *Arabidopsis* post-embryonic roots (Fridman *et al.*, 2021). However, how does the root's characteristic tapering shape get restored after injury?

When the root tip of an *Arabidopsis* seedling is surgically removed (resected) from a limited distance from the stem cell niche (SCN), it regenerates through a process of cell proliferation and repatterning, ultimately restoring its cell layers, spatial organization, and tapering morphology (Xu *et al.*, 2006; Birnbaum & Alvarado, 2008; Efroni *et al.*, 2016; Durgaprasad *et al.*, 2019). This repair process is likely different from the one that builds the root during development. During embryogenesis, the root forms at the basal pole of a single cell, whereas during regeneration, it is restored through the coordinated activity of multiple cell files, each with distinct identities. Previous studies on root tip regeneration have focused on genetic, hormonal, and molecular factors (Sena *et al.*, 2009; Efroni *et al.*, 2016; Durgaprasad *et al.*, 2019; Canher *et al.*, 2020; Mathew & Prasad, 2021). However, the role

of cell geometry in root tip regeneration, and plant wound repair in general, remains largely unexplored. This is an important area of research, as cell geometry changes are crucial during wound healing in animals as well. For instance, in *Drosophila* embryos and wing discs, cells near the wound edge undergo deformation, elongating to seal the wound and promote healing (Razzell *et al.*, 2014; Tetley *et al.*, 2019; Ton *et al.*, 2024).

In this chapter, I investigate how *Arabidopsis thaliana* roots restore their tapered shape following tip excision and suggest a two-step process guided by cell geometry. I found that the first step involves the generation of specific cell geometries through local cell shape changes. The second step uses these specific cell geometries to establish arrangements, such as a unique diagonal division plane, which facilitates the tapering of regenerating roots. Disrupting this cell geometry, either physically or genetically, impairs tapering and prevents complete restoration. This unprecedented shape-forming mechanism shows how local cell geometries drive tapering and provide fresh insights into plant organ shape regeneration. This previously unknown shape-forming mechanism highlights how local cell geometries drive tapering in regenerating roots, offering fresh insights into the processes underlying shape formation in plant organs.

VII.2 RESULTS

VII.2.1. Generation of specific cell geometries during root tip regeneration

The shape of a root can be conceptualized as a combination of three elementary structures: a cylinder extending from the transition zone through most of the meristem, a cone-frustum where the cylinder tapers over its distal portion, and finally, a cone or

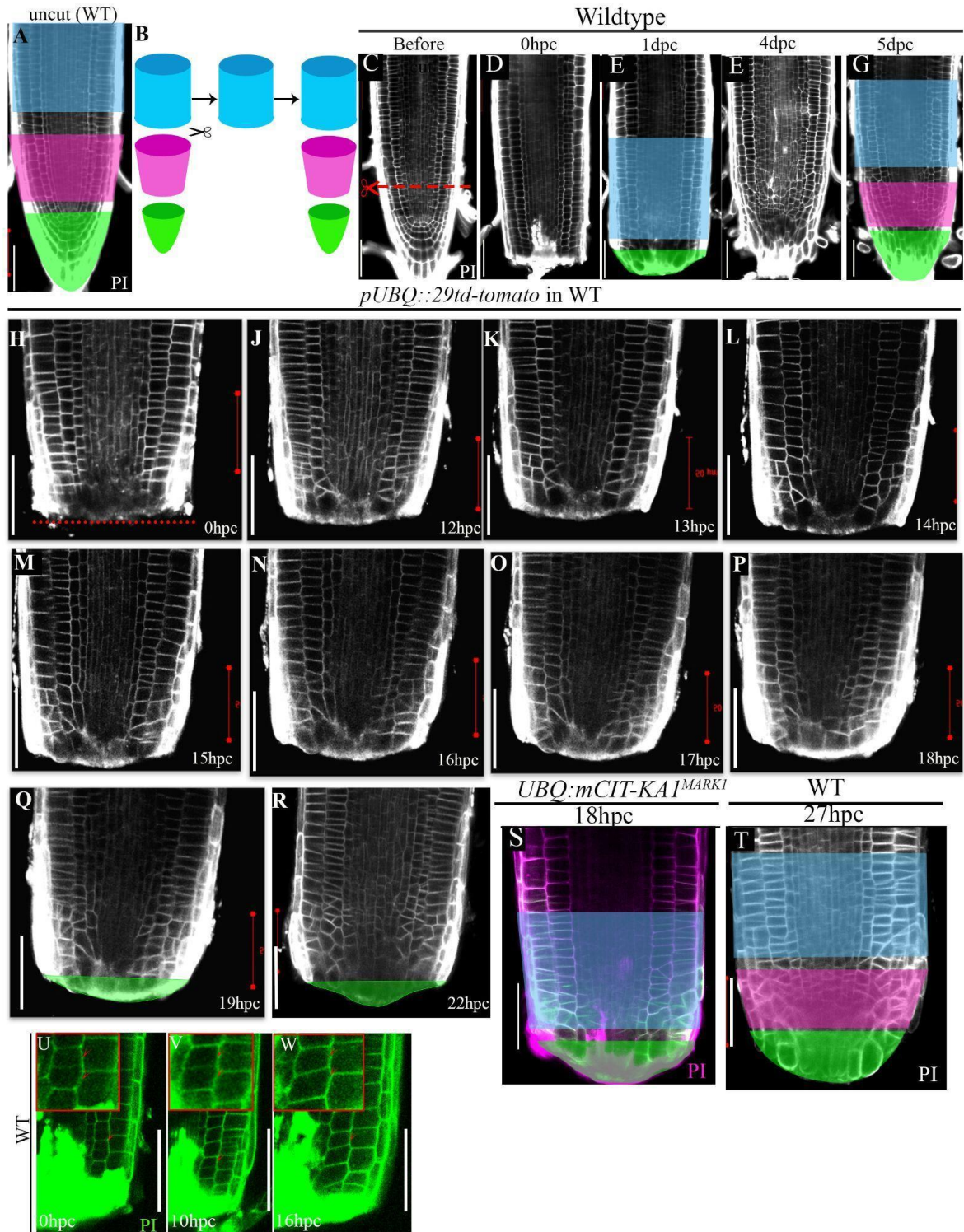


Figure. 7.1: Deformation of cells into rhomboid shapes during restoration of root tip. (A) Deconstruction of shape of the *Arabidopsis thaliana* root into three elemental shapes: cylinder (blue), cone frustum (magenta) and ellipsoid hemisphere (green). (B) Schematic representation of all the elemental shapes during root tip restoration. (H-R) Representative confocal time lapse image of regenerating wildtype roots tips marked with *UBQ::29tdtomato* at 0hpc-22hpc. Red dotted line: cut end which is flat. Green : generation of hemisphere at the cut end. Note that the rhomboid cells do not appear before 8hpc. (S-T) Confocal-based time-lapse imaging of a representative regenerating root tip post-resection in Columbia-0 *Arabidopsis thaliana*. Initially (18hpc), the root appears like a cylinder (blue) capped by a hemisphere (green)(S), and only later (27hpc) all the three elemental shapes are restored (T). (U-W) Representative confocal time lapse image showing that the cells that undergo deformation to rhomboid shape were cuboidal cells that were present at the time of cut. Scale bars: 50µm.

hemisphere at the columella. Here, I sought to simplify the complexity of root morphology by breaking it down into these fundamental shapes (Figure 7.1.A). Studies show that roots with just a cylindrical shape and a conical tip lack efficient soil penetration, highlighting the importance of the tapering (Mishra *et al.*, 2018). During root tip resection, I removed this tapered section (≈ 210 µm from the fourth tier of columella), leaving the root as a straight, vertical cylinder with flat end (Figure 7.1.B - 7.1.G). The cells immediately above the cut end undergo rapid cell proliferation to regenerate a new tip (Lee *et al.*, 2023); however, successful regeneration requires not only wound closure and proliferation but also the restoration of its original tapered shape. Restoring the original shape involves the reformation of three key shapes: the cylinder, which gradually tapers into a cone-frustum, with a conical or hemispherical cap at the tip (Figure 7.1.B). The generation of the cone frustum reflects the initiation of tapering, which I observed by 1 day post-cut (dpc), when the originally cylindrical root begins to gradually narrow (Figure 7.1.E). Consistent with prior studies (Sena *et al.*, 2009; Efroni *et al.*, 2016; Durgaprasad *et al.*, 2019; Canher *et al.*, 2022), I found that the resected root fully regained its original form by 5-dpc (Figure 7.1.G).

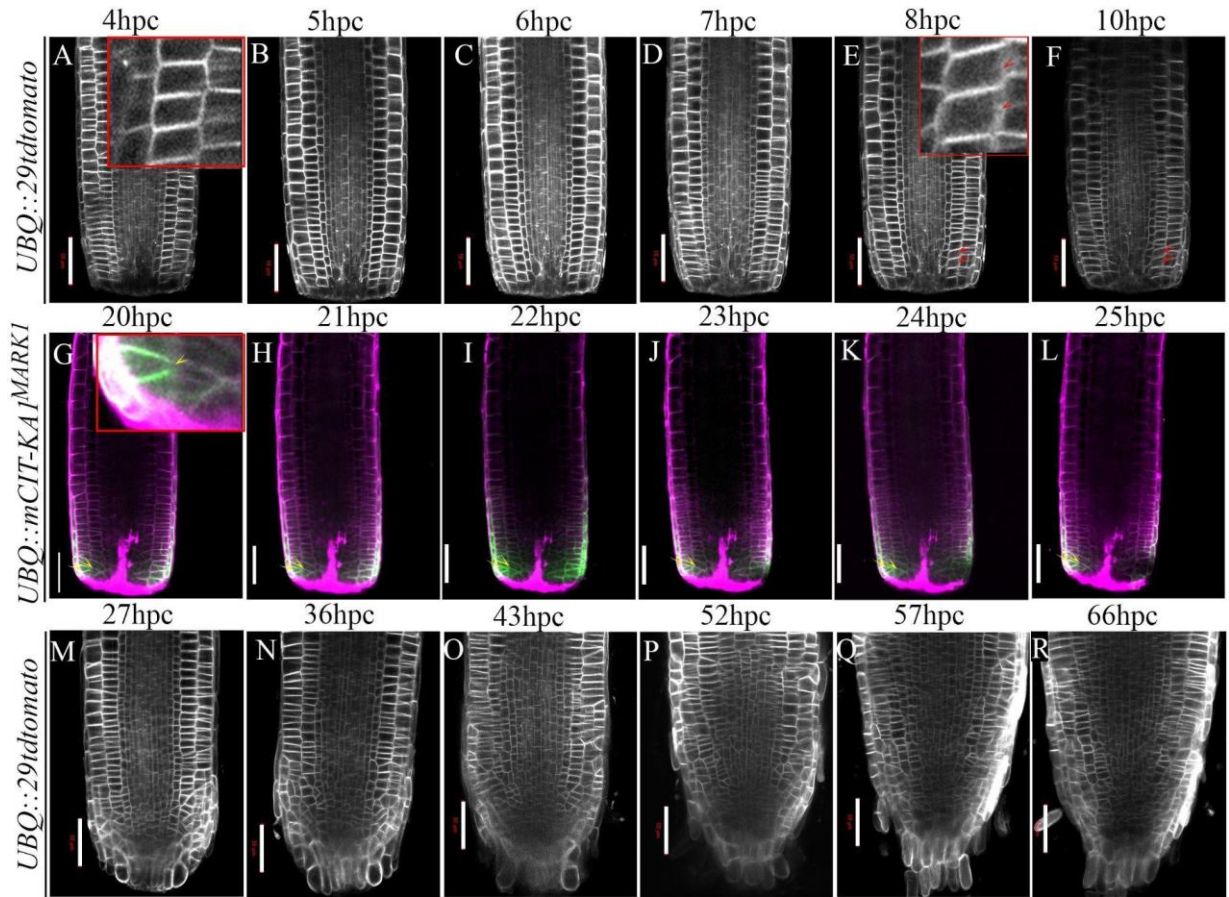


Figure 7.2: Rhomboid cells are temporally specific. (A-R) Representative time lapse imaging of regenerating root after low cut. Rhomboid cells first appear at 8hpc and disappear at 43hpc. White: *UBQ::29tdtomato*. Magenta: PI. Green: *mCIT-KA1^{MARK1}*. Scale bars: 50µm.

However, the mechanism driving the transition of the resected root from a vertical cylinder to an inclined, tapering form remains unclear. To investigate, I analyzed the overall root tip shape and individual cell behavior above the cut end at 1-hour intervals (Figure 7.1.H - 7.1.R, Figure 7.2). I found that within 12–19 hours post-cut (hpc), the flat cut end of the cylindrical root bulged outward, forming a convex boundary. As a result, the root initially took on the appearance of a cylinder capped by a hemisphere, marking the first phase of shape restoration (Figure 7.1.Q - 7.1.S). This was soon followed by the gradual narrowing of the cylindrical

root. At this point, the shape resembled a cone frustum placed between the cylinder and the hemisphere, marking the second phase of shape restoration (Figure 7.1.T). The onset of the cone frustum marks the initiation of tapering.

Notably, we identified a critical 10–24 hpc window, starting before tapering, during which 36.75% of the cells just above the cut end adopt a rhomboid-like shape, deviating from the typical cuboidal shape seen in uncut roots at the same location (Figure 7.3.A - 7.3.F). Interestingly, this geometric deformation primarily occurred in cells present at the time of the cut (Figure 7.1.U - 7.1.V). While rhomboid cells continued to appear after tapering began, their emergence peaked during the critical window. By 43-hpc, these rhomboid cells were completely undetectable, indicating that the deformation was transient (Figure 7.2.O).

To delve into the spatial distribution of these geometrically distorted cells, I measured the cell's exterior deformation angles (γ , γ' , ε , ε')—the angular changes at the top and bottom outer edges of cells in the meristematic zone—at 19 hpc (The deformations were most prominent at ≈ 19 -hpc) (Figure 7.4.A - 7.4.A). These angles reflect the extent of cell shape deformation. In cuboidal cells, the outer deformation angles are typically 0° , and deviations indicate geometric distortion. Rhomboid cells had increased exterior deformation angle ($\approx 5^\circ$; median), with the highest angles (up to 20°) (Figure 7.4.D - 7.4.E). Interestingly, the highest number of cells undergoing geometric deformation was observed in the cortex (Figure 7.4.F). Rhomboid cells in the cortex were more consistent in appearance and persisted for significantly longer periods compared to those in the endodermis. These deformed cells were concentrated within $70\ \mu\text{m}$ of the cut end, which I called 'Zone A^{cut}'.

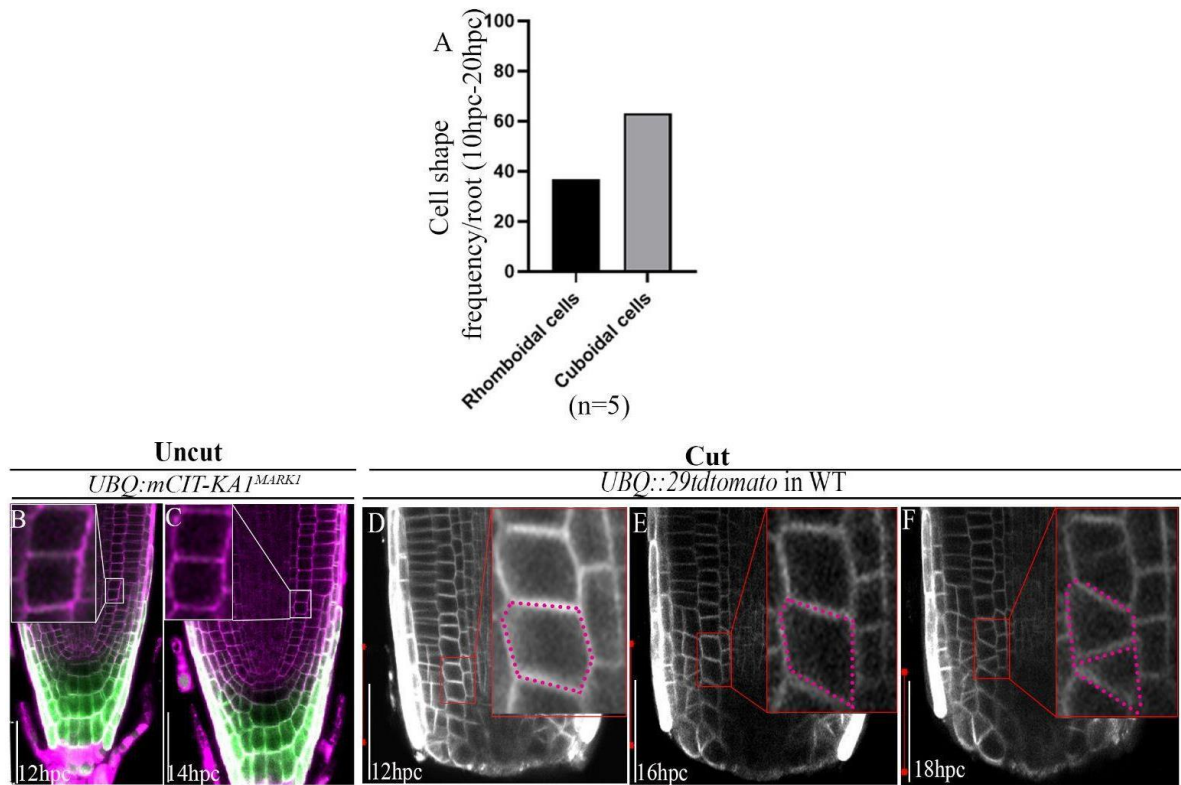


Figure. 7.3: Resection induced rhomboid cells are generated only in resected root tips. (A) Graphical representation of number of rhomboidal cells and cuboidal cells at 70 μ m from the cut end at 10hpc- 20hpc (n=5). (B-F) Representative confocal images from uncut and cut roots showing that deformation of cuboid cells to rhomboid occur in resected roots. n= sample number. Scale bars: 50 μ m. Error bars: S.E.M.

The corresponding region in uncut roots was termed 'Zone A^{uncut}'. The meristematic regions above these zones were labeled 'Zone B^{cut}' and 'Zone B^{uncut}', respectively. (Figure 7.4). Notably, the rhomboid cells were exclusive to Zone A^{cut}, indicating it was a feature induced by resection. Hence, I renamed Zone A^{cut} as the "Resection-Induced Geometrically Deformed" (RIGED) zone (Figure 7.4.B - 7.4.C). The RIGED zone is spatially and temporally confined, with its cells inclined toward the apex of the regenerating root tip (Figure 7.4.G). The alignment of these deformed cells mirrors the slope of the regenerating

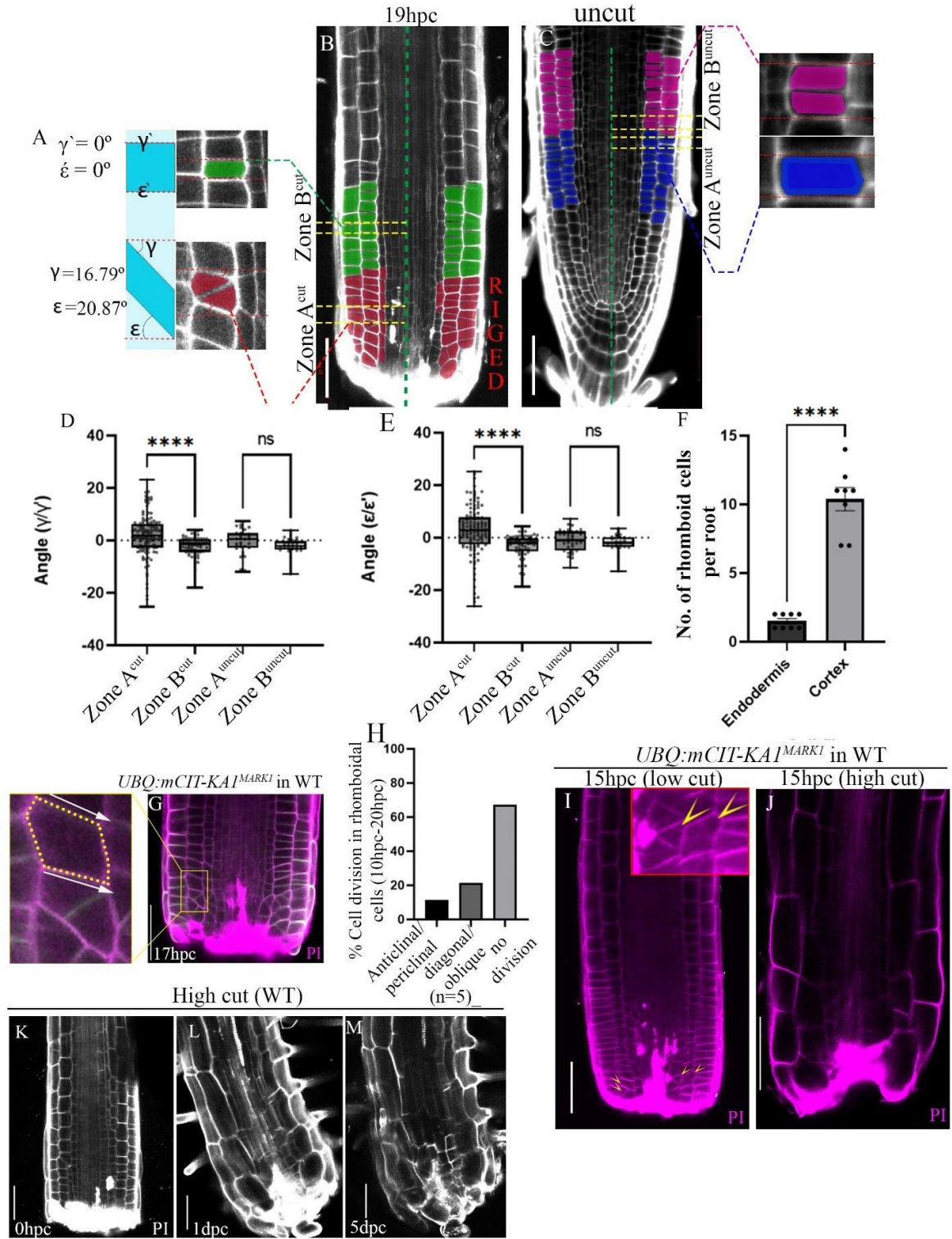


Figure. 7.4: Resection induced rhomboid cells are spatially specific. (A) Schematic representation of external deformation angles in a cuboidal cell (γ' , ε') and a rhomboid cell (γ , ε) (left). Representative confocal image of a cuboidal and a rhomboid cell in a regenerating root tip, where the $\gamma = 16.79^\circ$ and $\varepsilon = 20.87^\circ$ (right). Red dotted lines: parallel lines perpendicular to the root axis. **(B-C)** Representative live images of a 19 hpc regenerating root tip (left) and an uncut root tip (right) showing zones A^{cut} , B^{uncut} , A^{uncut} , and B^{uncut} . Red cells: Zone A^{cut} (RIGED Zone), within 70 μm from the cut end, comprising mainly rhomboid cells. Green cells: Zone B^{cut} , above 70 μm from the cut end, mainly cuboidal cells. Blue cells: Zone A^{uncut} , counterpart of A^{cut} in uncut roots, mainly cuboidal cells. Magenta cells: Zone B^{uncut} , counterpart of Zone B^{cut} in uncut roots, mainly cuboidal cells. Yellow dotted lines: parallel lines perpendicular to the root axis (green dotted line). White: PI stain. **(D-E)** Graphical representation of the deformation of cells in all four zones, where deformation is significant only in the RIGED Zone (zones A^{cut}). **(F)** Graphical representation of the number of rhomboidal cell per root in cortex v/s endodermis (n=8; Mann-Whitney U test; ****p-value < 0.0001). **(G)** Representative live image of a root at 17hpc. Inset shows a rhomboidal cell tilted towards the root apex. White arrows: direction of tilt. **(H)** Graph showing the types and frequencies of cell division (number of cells, x= 61; number of roots n =5) in regenerating roots from 10 hpc–20 hpc. **(I-J)** Representative confocal time lapse image of wildtype roots in response to low cut (I) and high cut (J) at 15hpc. Yellow arrowhead denotes the rhomboid cells. **(K-L)** Representative confocal time lapse image of wildtype roots in response to root tip resection at 280–300 μm from fourth tier of columella (high cut) at 4hpc–10hpc. Red arrowhead denotes the rhomboid cells. Note that the rhomboid cells do not appear before 8hpc. n= sample number. Scale bars: 50 μm . Error bars: S.E.M

root. I found that 21.31% of the geometrically deformed cells in the RIGED zone undergo oblique or diagonal divisions, while (11.47%) showed anticlinal or periclinal divisions, while the remaining cuboidal cells underwent periclinal or anticlinal divisions (Figure 7.4.H). To track the emergence of division planes, I used confocal imaging with *pUBQ::mCIT-KA1^{MARK1}* (Platre *et al.*, 2018), which marks newly formed division planes due to its sensitivity to electronegativity, as newly formed walls are significantly more electronegative than old ones. Importantly, no rhomboid cells or diagonal divisions were observed when cuts were made higher (250–300 μm from the fourth tier of the columella; Figure 7.4.I). Consistent with what was previously shown by Durgaprasad *et al* (2019), I also

found that regeneration does not occur in response to high cuts (Figure 7.4.J - 7.4.L). This indicates that the formation of rhomboid cells and diagonal divisions is specific to regeneration initiation, rather than a general response to resection or wound closure.

VII.2.2. Non-uniform growth of the neighboring cells generates the rhomboid cell geometries

I next investigated what triggers the deformation of cells in the RIGED zone into rhomboid shape and the formation of a hemisphere-shaped cap at the cut end. Reasoning that cell geometries are influenced by mechanical strain resulting from spatial differences in growth (Dorothee *et al.*, 2022), I hypothesized that the growth heterogeneity within the regenerating root could drive the deformation of cells in the RIGED zone into rhomboids. Since the highest number of rhomboid-shaped cells are observed in the cortex (Figure 7.4.F), situated between the faster-growing stele and the slower-growing epidermis, I proposed that the stele and endodermis 'pull' the stele-ward side of cortical cells, while the opposite side remains relatively stationary due to the slower growth of the epidermal cells (Figure 7.5).

To test this, I first monitored the growth rate of cells in the RIGED zone at 10 hpc-18hpc– the period rhomboid cells were most prominent– and compared it with the same region in uncut roots for the same period (Figure 7.6.A - 7.6.F). It should be noted that I considered growth as a combination of both cell expansion and growth in cell lineages of dividing cells. I observed differences in growth rates among the longitudinal cell files of the regenerating root tip: cells in the stele and endodermis grew faster than those in the cortex, while cells in

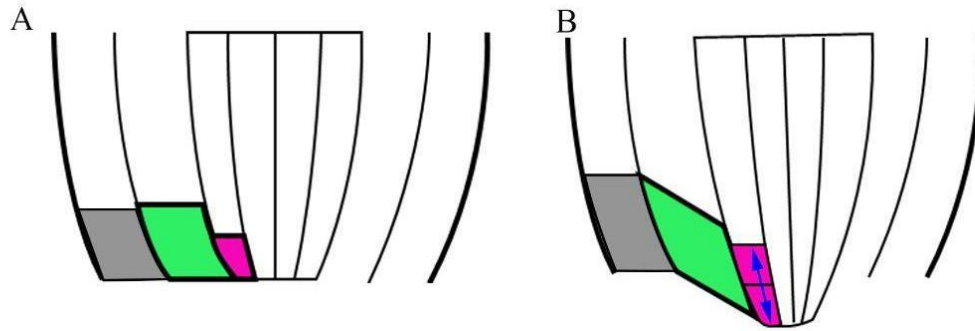


Figure. 7.5: Schematic representation of the hypothesis that faster growth in the stele and slower growth in the epidermis caused cuboid shaped cortical cell to deform to rhomboid. (A-B) Pink: stele (inner cell), green: cortex (middle cell), grey: epidermis (outer cell).

the epidermis grew more slowly than those in the cortex (Figure 7.6.A - 7.6.E). This spatial growth difference was consistent with a previous study that showed pronounced growth heterogeneity between cell files during root regeneration, with cell division being fastest in the stele and endodermis, and slowest in the epidermis between 10 hpc and 18 hpc (Rahni *et al.*, 2024). This suggests a link between the growth rate differences in neighboring cell files and the formation of distinct cell geometries.

A series of 2-D finite-element simulations of a regenerating root tip were conducted using the Morphodynamics (MDX) framework to investigate whether differences in growth rates between cell files can generate rhomboid cells. This was carried out in collaboration with Jeroen Saccheri, Dr. Monica Garcia, and Prof. Kirsten Ten Tusscher; Utrecht University, Netherlands. In the simulations, all cells within a limited distance from the cut end were simulated to grow, except for the first layer of cells, which corresponds to the dead

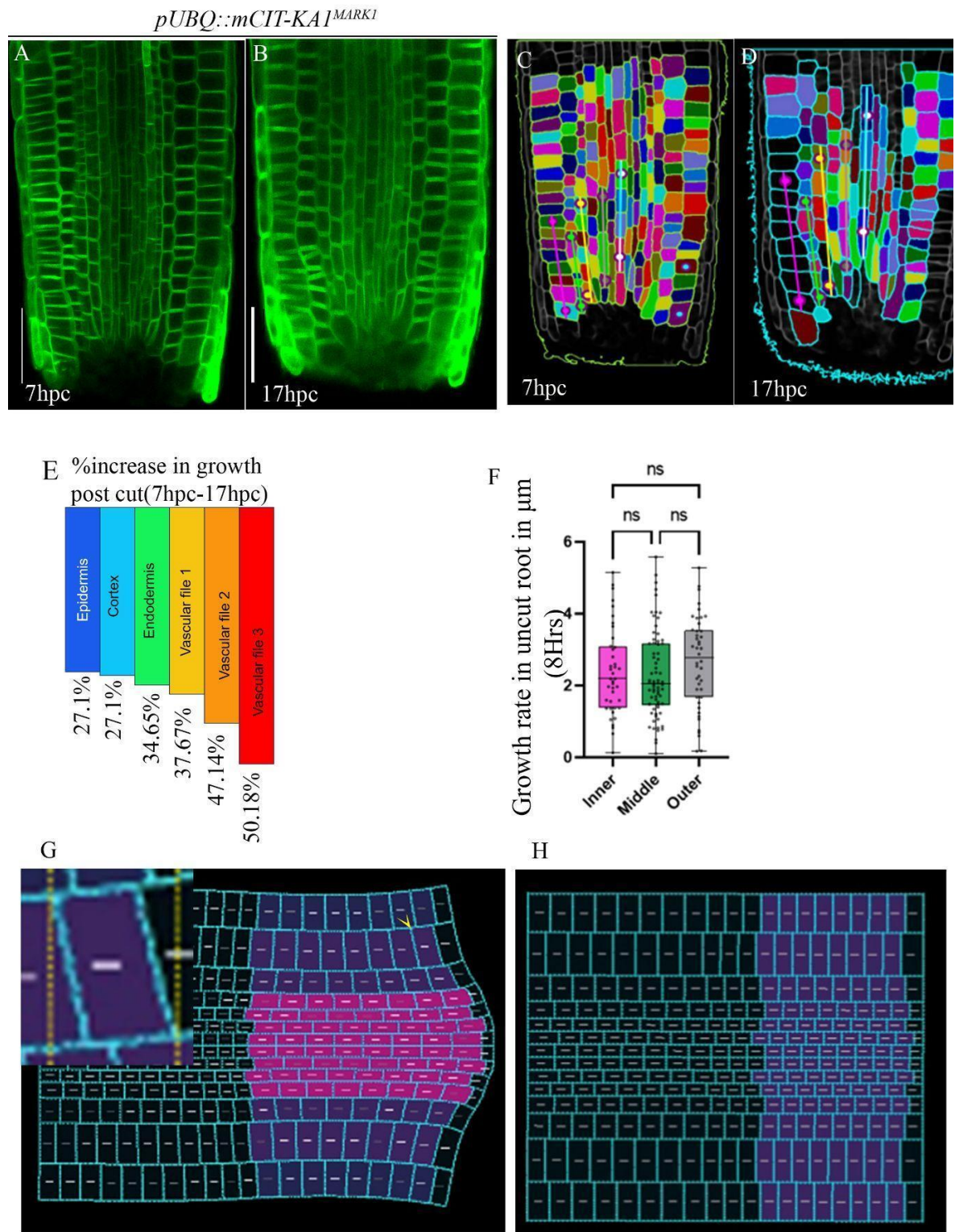


Figure. 7.6: Non- Uniform growth of the neighboring cells trigger the cell deformation. (A-B) Representative time lapse image of a root at 7hpc and 17hpc. (C-D) Mophograph-X

segmented root at 7hpc and 17hpc from A-B. **(E)** Graphical representation of the growth of each cell file in resected root from 7hpc-17hpc based on growth rate analysis in MGX. **(F)** Graphical representation of the growth of each cell file in uncut root for 8 hours (x=120 cell; n=4 roots; Kruskal-Wallis test followed by Dunn's multiple comparisons test; ns p-value=0.9177 between inner and middle cells; ns p-value > 0.999 between inner and outer cells; ns p-value > 0.999 between middle and outer cells). **(G)** Frame capture of a simulation where stele (magenta) growth in the regenerating root is higher and epidermis (navy) is slower than the rest of the cell files. Yellow arrowhead indicates the rhomboid cell in the cortex (middle; purple). The black cells near the cut end represent non-growing cells (to recapitulate damage). The black cells at the other end of the simulated tissue are also not growing. **(H)** Frame capture of a simulation where stele growth in the resected root is the same as the rest of the cell files. There is no formation of rhomboid cells indicating growth rate differences are essential for rhomboid cell formation. n= sample number. Scale bars: 50µm. Error bars: S.E.M

cells at the cut end. The first simulation incorporated the experimentally observed faster growth in the stele and slower growth in the epidermis (Figure 7.6.G). The second simulation was the control which assumed equal growth across all cell files, with no growth rate differences between the stele and epidermis (Figure 7.6.H). Unlike the control, the simulations close to the experimental data predicted the generation of rhomboid cells, suggesting that faster stele growth and slower epidermal growth contribute to rhomboid cell formation. These simulations not only identified the cause of rhomboid cell formation but also demonstrated the transformation of the flat boundary at the cut end into a curved, convex boundary, ultimately forming a hemisphere-like cap (Figure 7.6.G). As anticipated, in the control simulation where there was no unequal growth, the boundary was straight and flat (Figure 7.6.H).

I reasoned that the *woodenleg* (*wol*) mutant, which has reduced vascular initials and lacks proper stele proliferation (van den Berg *et al.*, 1995; Mähönen *et al.*, 2000; Ohashi-Ito & Bergmann, 2007), will not have the necessary faster growth in the steles, and therefore would fail to exhibit resection-induced geometric deformation. Preliminary results reinforce this notion, and further analysis into this mutant is ongoing in the lab. After cuboidal cells deform into rhomboid shapes, their anisotropic growth is essential for maintaining this form. I noticed that the rhomboid cells expand anisotropically, with their growth directed toward the root apex (Figure 7.4.G). This anisotropic growth of rhomboid cells likely involves cell wall remodeling and the polarized delivery of cell wall materials. To investigate this, I designed experiments and procured the necessary genetic backgrounds, and genetic analysis is currently underway in the lab.

In summary, the differential growth rates between the stele and epidermis drive both the deformation of cells into rhomboids and the formation of a hemispherical cap at the cut end. These processes together mark the first stage of root shape restoration during root tip regeneration.

VII.2.3. Rhomboid cell shape guides the oblique plane of division to restore the tapering shape of the root following excision of the tip

Next, I investigated the cellular mechanisms driving the second phase of root tip shape restoration: the tapering (Figure 7.7). While tracking cell behavior, I consistently observed that rhomboid cells undergo diagonal or oblique divisions, producing daughter cells with a triangular prism-like shape (Figure 7.7.C – 7.7.D). An important question is

whether this unusual division orientation occurs alongside the formation of rhomboid cells or results from it. Previous models, such as Errera's Rule, propose that the division plane is perpendicular to the longest axis of the cell, with the new wall forming along the shortest path to produce two daughter cells of equal size (Figure 7.7.A). In the case of rhomboid-shaped cells, the longest axis corresponds to the major diagonal, connecting the two farthest-apart corners, while the shortest axis is the minor diagonal, which is perpendicular to the major diagonal (Figure 7.7.B). So, in theory it is plausible that the diagonal division in the rhomboid cells is an outcome of the cell shape.

To investigate how rhomboid cell shape may result in diagonal divisions, *in silico* simulations were performed to predict the division orientation in rhomboid-shaped cells. These simulations utilized a microtubule (MT) framework, which had previously been applied to model division planes in square-shaped cells (Chakraborty *et al.*, 2018a), and were carried out in collaboration with *Bandan Chakraborty at IISER-TVM*. This framework simulates pre-prophase MT dynamics on various cell shapes, incorporating MT properties like nucleation, dynamic instability, and MT interactions (Chakraborty *et al.*, 2018a) (Figure 7.7.E). Additionally, the framework accounts for 'edge catastrophe,' which refers to the depolymerization of microtubules when they cannot cross from one cell face to the adjacent face due to sharp cell edges. These sharp edges arise from smaller angles between adjacent faces, increasing the likelihood of edge catastrophe. To avoid this, microtubules form an array away from the edges. Under this framework, the simulations reached a steady state, forming a cortical MT array. The orientation of the MT array was measured by the R vector which is perpendicular to the cortical MT array (Chakraborty *et al.*, 2018b). In this study,

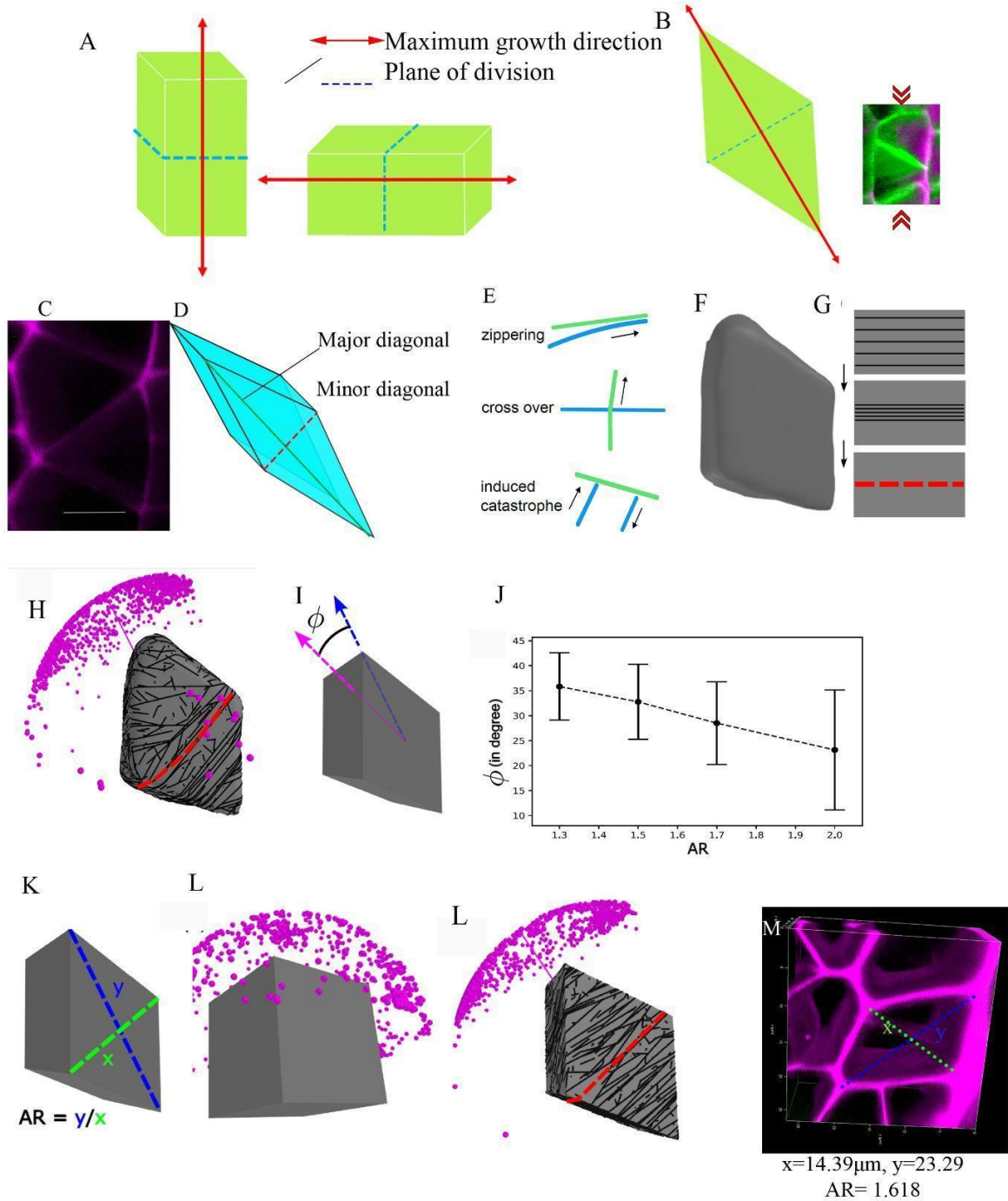


Figure. 7.7: Rhomboid shape guides the diagonal plane of division. (A-B) Schematic representation of impact of cell geometry on plane of cell division. (C) Representative confocal based 3D reconstitution of a rhomboid cell with diagonal division. Scale bar: 5μm. (D) Schematic of rhomboid cell showing major and minor diagonals. (E) Schematic

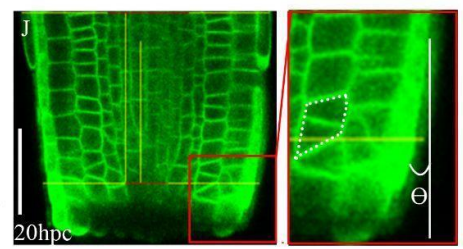
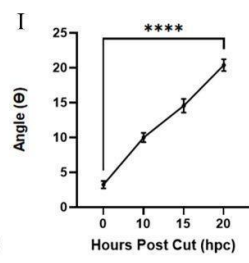
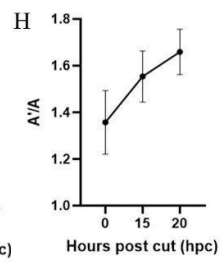
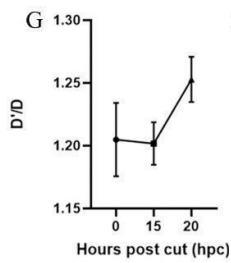
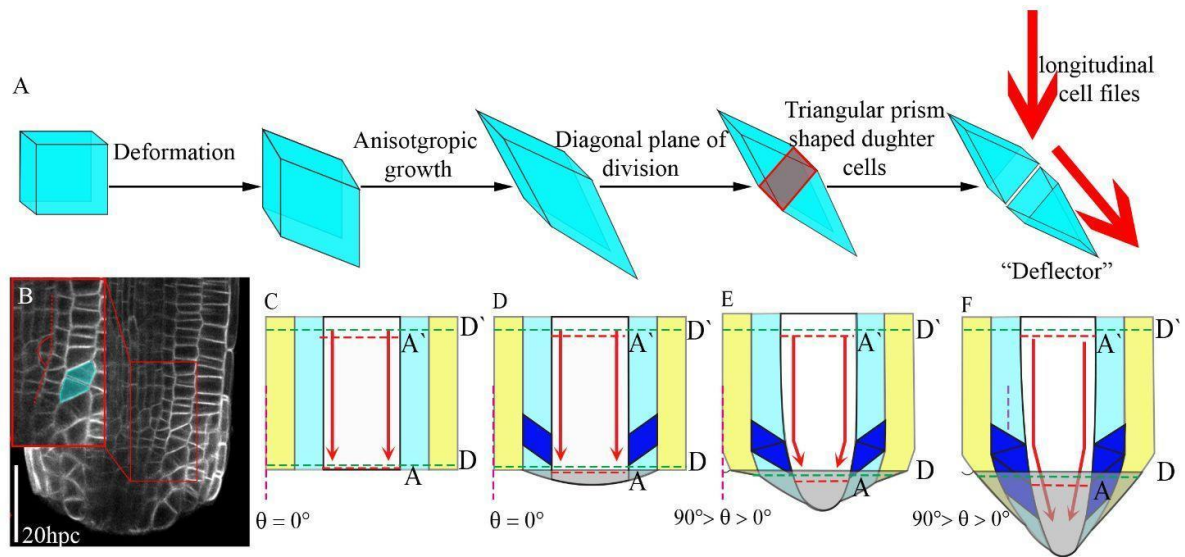
representation of MT-MT interaction rules. MTs colliding at shallow angles ($\leq 40^\circ$) undergo zippering, crossover, or induced catastrophe with approximately equal probability. **(F)** Representative rhomboid cell (3D reconstruction by merging the daughter cell pair in MGX). **(G)** Schematic representation of cell division orientation via MT array. MTs, through dynamic interactions, form an ordered array (top). The MT array then forms a ring-like structure called Pre-Prophase Band (PPB, middle). The position and orientation of the PPB determine the corresponding position and orientation of the division plane (bottom). **(H)** Simulation of MT dynamics on the surface of experimentally obtained rhomboidal cell shape showing oblique orientation MT array vector tips R (magenta dots). The arrow vector (magenta arrow) represents the mean orientation of the associated MT array. **(I)** Schematic representation of the deviation angle of the division plane from the oblique division (Φ), the angle between R (magenta arrow) and the principal axis (blue arrow) of the rhomboid cell shape. **(J)** Graphical representation of (Φ) upon AR increase. **(K)** The cell aspect ratio is defined as the ratio of the magnitude of the major axis (y-dimension) and minor axis (x-dimension) of the cell. **(L)** Simulation of MT dynamics on a cuboidal cell shape, i.e., with AR = 1, showing random orientation MT array vector tips. **(M)** Simulation of MT dynamics on an experimentally equivalent idealized rhomboid cell shape showing oblique orientation MT array vector tips. **(N)** Representative rhomboid cell with AR ≈ 1.62 .

rhomboid cell shapes from microscopic images of regenerating root tips were first extracted (Figure 7.7.F), followed by simulation of MT dynamics in the extracted cells, accounting for both MT-MT and MT-substrate interactions (Figure 7.7.G). The simulations predicted diagonal MT array orientation, matching the experimentally observed diagonal and near diagonal division planes (Figure 7.7.H).

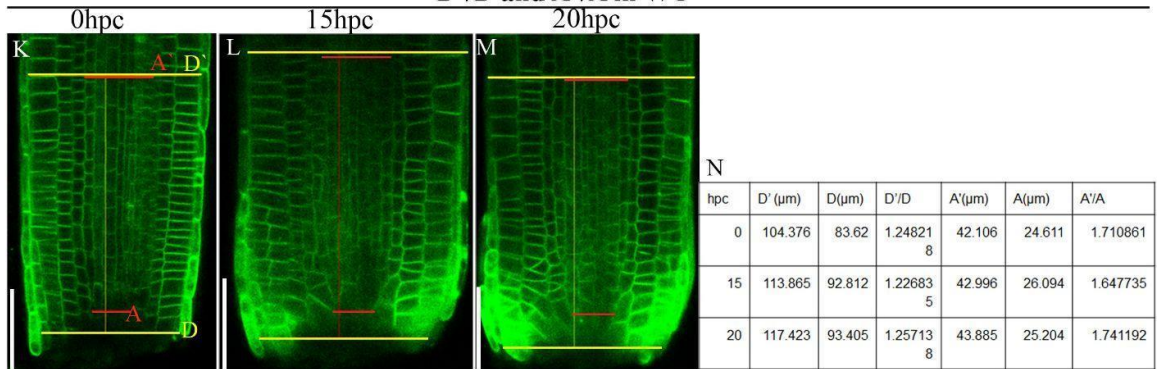
Next, I quantified the precision of the predicted diagonal division by measuring the angle (ϕ) between the cell's principal (longest) axis and the R vector, which is perpendicular to the microtubules. This angle (ϕ) indicates the deviation of the division orientation from the predicted diagonal division (Figure 7.6.I). Notably, ϕ was found to be inversely proportional to the cell's aspect ratio (AR), defined as the ratio of the longest axis to the shortest axis (Figure 7.7.J - 7.7.K). In theory, a higher AR corresponds to a greater degree

of rhomboidness. For a cell template with $AR = 1$, simulations predicted random division planes (Figure 7.7.L). However, as the AR increased to 1.66—matching the aspect ratio observed in rhomboid cells experimentally— ϕ decreased. This reduction in ϕ coincided with microtubule (MT) arrays aligning diagonally, favoring a diagonal division plane (Figure 7.7.M - 7.7.N).

Having established that rhomboid cell shapes lead to diagonal divisions, where one daughter cell is displaced outward and the other toward the tip, I speculated that these divisions act as a "deflector," redirecting vertically growing longitudinal cell files and guiding them onto an inclined trajectory (Figure 7.8.A). I observed that when longitudinal cell files encounter rhomboid cells undergoing diagonal divisions, they deviate from their straight vertical path and take a subtle turn onto an inclined trajectory (Figure 7.8.B). This deviation was critical for the narrowing of the cylindrical root into a cone frustum shape, a key step in restoring the root tip (Figure 7.8.C - 7.8.F). To investigate this, I analyzed the relationship between diagonal divisions and root tapering. Several hours after the first diagonal divisions at 12hpc, the root began to taper by 20hpc. The initiation of this tapering coincided spatially with the location of the diagonally divided cells (Figure 7.8.C - 7.8.Q). Quantitatively, this was captured by the D'/D ratio, which represented the root's diameter at the transition zone (D') relative to the diameter near the cut end where diagonal divisions occurred (D). The D'/D ratio increased from 1.205 ± 0.082 at 0hpc to 1.253 ± 0.050 at 20hpc, indicating root narrowing (Figure 7.8.G, 7.8.K - 7.8.N). A similar pattern was observed in the stele, where the A'/A ratio—the stele diameter at the transition zone (A') relative to its



D'/D and A'/A in WT



θ in WT

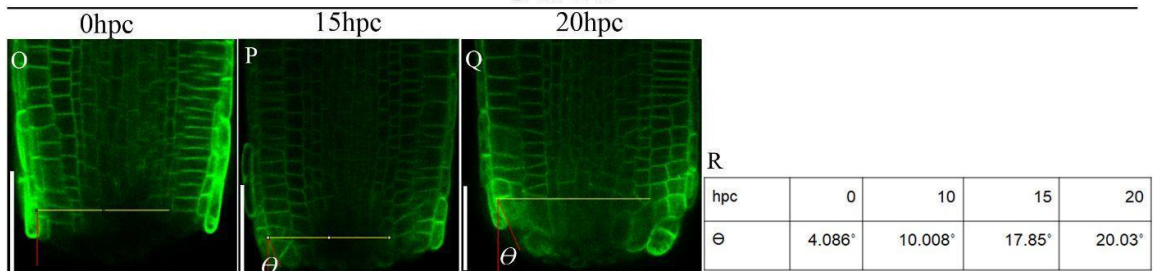


Figure. 7.8: Rhomboid cell shape guides the oblique plane of division to restore shape of the root tip. (A) Schematic representation theorizing the stages of a deformed cell. The cuboidal cell first deforms into rhomboid, which then grows anisotropically and undergoes diagonal division to produce a pair of daughter cells shaped like triangular prisms. The daughter cells then proceed to act like “deflectors” to prompt the vertically growing longitudinal cell files to take a subtle turn and continue along an inclined trajectory. (B) Representative live image showing deviation of longitudinal files upon encountering diagonally divided rhomboid cell (cyan). (C-F) This schematic representation depicts the progression of shape recovery during root tip regeneration, focusing on how rhomboid cell deformation and alignment contribute to the tapering process. Immediately after the root tip is resected, the cut end remains flat. θ refers to the angle of tapering or deviation of the root tip during regeneration. Initially, the regenerating root tip is straight, with no tapering ($\theta = 0^\circ$), meaning that the cells align vertically (C). Yellow represents the epidermis (outer cell file), blue represents the cortex/endodermis (middle cell file), and white indicates the stele/pericycle (inner cell file). The red arrow shows the growth direction of the inner cell file. As the inner cell files grow faster, cells in the middle cell file undergo geometric deformation into a rhomboid shape (D). Grey shows the regenerated portion below the cut end. The rhomboid cells (dark blue) then undergo diagonal divisions, producing daughter cells that are inclined toward the slope of the root apex rather than aligned vertically. This causes the inner cell files to deviate from their original vertical growth axis and take a subtle turn. As this occurs, θ increases, indicating that the tip is no longer straight but is sloped (PI). Tapering becomes more pronounced as additional cells deform into the rhomboid shape and continue undergoing diagonal divisions (F). Red dotted vertical lines indicate the original growth axis of the root, while green dotted horizontal lines denote the diameter of the root, spanning from epidermis to epidermis. Red dotted horizontal lines mark the diameter of the stele. (G) Graph depicting D'/D , the ratio of the root's diameter at the transition zone (D') to its diameter at the position of diagonal division near the cut end (D) from 0 hpc-20 hpc (number of roots, $n=8$; Paired t-test; ns p-value = 0.1172). (H) Graph depicting ratio of the stele diameter at the transition zone (A') to that at the position of diagonal division near the cut end (A) from 0 hpc-20 hpc ($n=8$; Paired t-test; *p-value = 0.025). (I) Graph depicting the angle of tapering (θ), which denotes the degree of deviation of the regenerating root from vertical (straight) alignment from 0 hpc-20 hpc ($n=8$; Paired t-test; ****p-value < 0.0001). (J) Representative confocal image showing tapering occurs at the position of diagonally divided rhomboid cells. (K-N) Representative time lapse image (K-M) and table (N) indicating the D'/D and A'/A . (O-R) Representative time lapse image (O-Q) and table (R) indicating θ . n = sample number. Scale bars: 50 μ m. Error bars: S.E.M.

diameter near the cut end (A)—increased from 1.554 ± 0.244 at 0hpc to 1.659 ± 0.217 at 20hpc (Figure 7.8.H, 7.8.K - 7.8.N).

These structural changes aligned with an increase in the angle of tapering (θ), which measured the deviation of the regenerating root from vertical growth. Initially, at 0hpc, $\theta \approx 3^\circ$, indicating near-vertical alignment. By 18-20 hpc, θ increased to $\approx 20^\circ$, marking the onset of tapering (Figure 7.7.I - 7.7.J, Figure 7.7.O - 7.7.R). It should be noted that, at 20-hpc, θ was measured at a position that coincided with the spatial location of the diagonal divisions. Together, these findings demonstrated longitudinal cell files deviated from their original vertical path and continued along an inclined trajectory. This deviation coincided precisely with the spatial position of the diagonally divided cells, underscoring their critical role in driving root tapering.

In summary, I show that the second phase of shape restoration is driven by the diagonal divisions of rhomboid cells, which serve as deflectors, redirecting vertically growing longitudinal cell files onto an inclined trajectory. This deviation is crucial for the narrowing of the root's diameter, initiating the tapering process.

VII.2.4. Root tapering relies on generation of rhomboid cells

To further cement my hypothesis that rhomboid-shaped cells are essential for tapering during root regeneration, I ablated 10–15 rhomboid cells from the RIGED zone at 12-14 hpc using targeted laser ablation (Figure 7.9.A). Since most of the rhomboid cells were from the cortex, I focused the ablation on cortical cells. It is important to note that I did

not ablate all rhomboid cells in the RIGED zone. Given the cylindrical, three-dimensional structure of the root, the rhomboid cells are arranged in a circular pattern around the stele. Due to technical limitations in accessing all of these cells with high resolution and to prevent excessive damage that could impair the root's regenerative ability, I restricted the ablation to clearly resolved cells in the middle plane (Figure 7.9.A - 7.9.J). Even with the removal of only a subset of the rhomboid cells, tapering was significantly delayed; the regenerating roots post ablation began tapering only by 3-dpc after ablation, in contrast to the control roots, which initiated tapering by 1-dpc and formed robust conical tips by 2-dpc (Figure 7.9.B - 7.9.G). This delay was evident at 2-dpc, when the ablated roots had a flat contour, in contrast to the tapered contour observed in the controls (Figure 7.9.D, 7.9.G).

Interestingly, despite the lack of tapering at 2-dpc, the expression pattern of the root stem cell regulator, WUSCHEL-RELATED HOMEODOMAIN 5 (WOX5) was unaffected and similar to that of control roots (Figure 7.9.F, 7.9.I). This indicates that the role of rhomboid cells in initiating root tapering is independent of WOX5-mediated stem cell regulation. It should be noted that the ablated roots still underwent cell proliferation, but began tapering only once new rhomboid cells replaced the ablated ones, eventually restoring the entire root tip morphology. In summary, the data from laser ablation demonstrate that the end of the root after resection remains flat in the absence of rhomboid cells and regains its tapering shape only after the rhomboid cells reappear. Thus, my findings indicate that rhomboid cell shape prompts initiation of tapering in regenerating root tips.

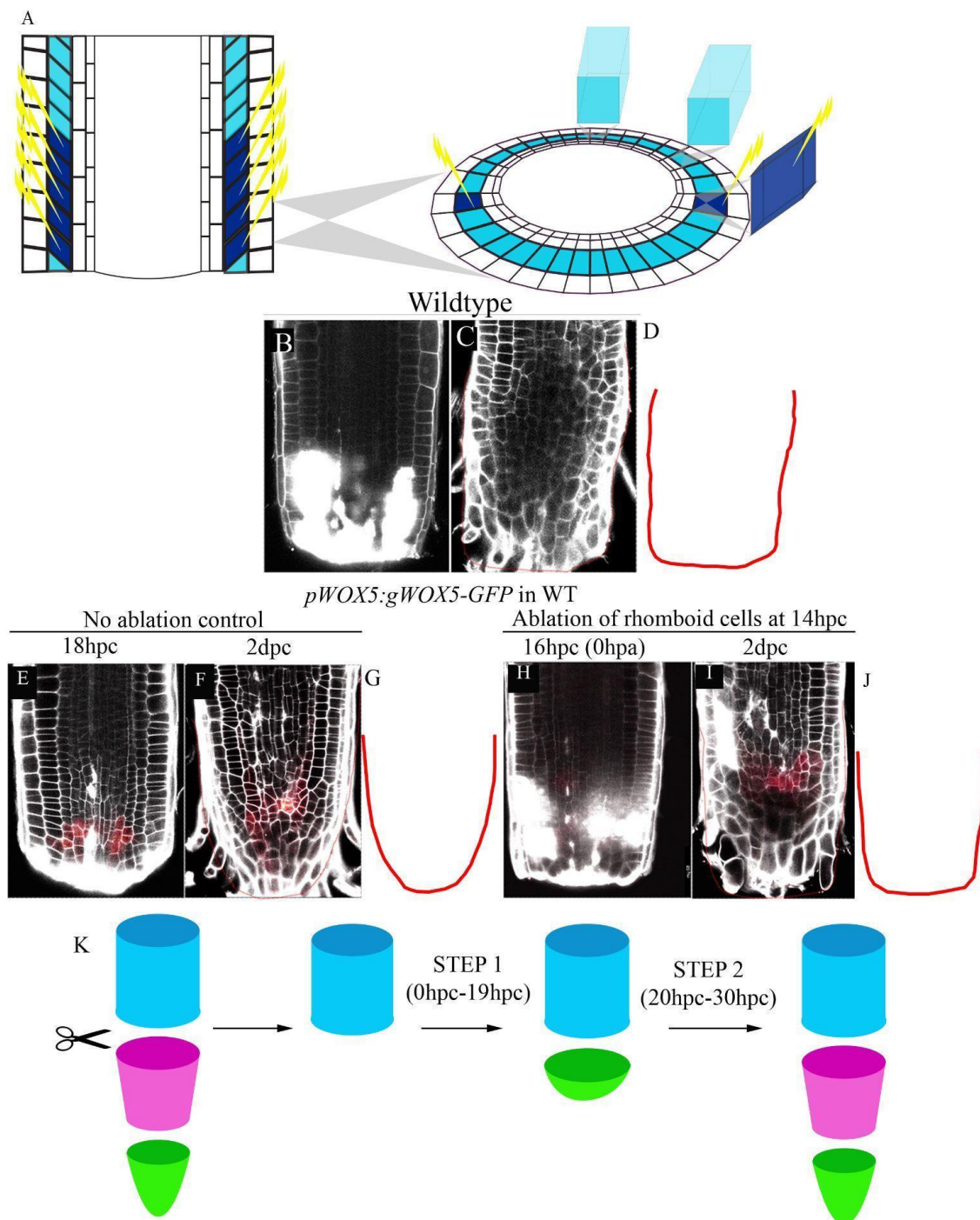


Figure. 7.9: Rhomboid cells are necessary for initiating the tapering during root tip restoration. (A) Schematic representation of rhomboid cell ablation in regenerating root tips. Left: Longitudinal section (L.S) showing ablation of five rhomboid cells on each side

of the stele. Right: Cross section (C.S) showing ablation of two rhomboid cells on opposite sides of the stele. Light blue: cortical cells. Dark blue: ablated cells. **(B-J)** Ablation of rhomboid cells. Laser-targeted removal of 10–16 rhomboid cells at 16 hpc impairs tapering initiation in resected root tips (B-D, H-J). By 2 dpc, the regenerating root tip without rhomboid cells remains flat (C-D, I-J), compared to the tapered shape in control roots F-G), where rhomboid cells remain intact. Expression of the root stem cell regulator, WOX5, remains comparable between the ablated roots (I) and the control (F). Red dotted/solid lines: contours of root tips at 2 dpc. White: PI stain. Scale bars: 50µm. **(K)** Schematic representation depicting the two steps of root tip restoration. Upon resection, the tapering part of the root is removed, leaving behind only a cylinder-shaped part. By 19hpc the boundary at the cut end bulges in a convex fashion such that the root appears like a cylinder capped by a hemisphere. This marks step 1. After 20hpc, the root begins to taper, such that the root appears like a cylinder which narrows into a cone frustum capped by a hemisphere. This marks step 2. Scale bars: 50µm.

Based on these observations, I suggest that the restoration of the root's tapered shape occurred in two steps. In Step 1, faster growth in the stele and slower growth in the epidermis deformed cortical cells near the cut end into rhomboid shapes and created a curved boundary at the cut end, transforming the root from a cylinder with a flat base to a cylinder capped by a hemisphere. In Step 2, diagonal cell divisions and the ensuing inclined trajectories of longitudinal cell files narrowed the root's diameter, initiating tapering and ultimately restoring all three key shapes of the root (Figure 7.8.K).

VII. 3. DISCUSSION

My study reveals how the interaction between growth rate differences, cell shape, and division orientation drives organ shape formation. Using *Arabidopsis thaliana* root tip regeneration as a model, I investigated the factors that restore the tapered shape of regenerating roots. Based on my experimental and computational data, I suggest a two-step model (Figure 4A) that outlines the cellular and tissue-wide events involved in this process.

In the first step, mechanical strain from uneven growth rates between neighboring cell files deforms cuboidal cells into rhomboid shapes. In the second step, diagonal cell divisions in these rhomboid cells and the resulting inclined growth of longitudinal cell files progressively narrow the root's diameter, initiating tapering.

It makes one wonder what causes the stele in resected roots to grow faster than the remaining cell files. The faster proliferation observed in the stele has been previously reported to be between 10-18 hpc, suggesting that factors triggering this rapid growth are activated before this period. One key player in this process appears to be auxin, which surges at the cut end by 6 hpc due to both local biosynthesis and accumulation (Canher *et al.*, 2020). This excess of auxin at the cut end likely needs to be stabilized. In uncut roots, an auxin reflux loop at the stem cell niche (SCN) at the distal tip stabilizes auxin levels (Blilou *et al.*, 2005). However, with root tip resection, the distal tip and SCN are removed, disrupting the reflux loop and destabilizing auxin levels. To restore stability, I propose that a new SCN must form distally. During a short 6-12 hpc window, auxin and cytokinin briefly overlap in the stele, marking the site for the new SCN (Efroni *et al.*, 2016). Based on this, I speculate that the dynamics of auxin and cytokinin likely trigger the rapid proliferation in the stele, helping to position the new SCN distally.

Could the pull from the fast-growing inner (stele) cell files alone explain the formation and maintenance of rhomboid-shaped cells? Although probabilistic, it is tempting to consider that this force might work in tandem with centripetal constriction from the outer (epidermis) cell files and cell death within the inner files to shape and facilitate the anisotropic growth of these rhomboid cells. While these possibilities remain untested, the

2D finite-element modeling, where increased stiffness in the epidermis was simulated to mimic the first phase of shape restoration, suggests a potential role for epidermal mechanics in this process, warranting further experimental exploration.

Thus, my study offers interesting insights on how the geometric, and mechanical factors integrate with the biochemical factors to restore the shape of a regenerating organ.

***Chapter VIII: Regulatory role of
PLETHORA 2, a plant specific
Transcription Factor in root tip
regeneration***

Mathew M.M et al., *unpublished*

VIII. 1. INTRODUCTION

"The whole is greater than the sum of its parts."

— Aristotle

Regeneration of the root tip post-excision is a multifaceted process that involves changes in cell identities, transient fate changes, cell proliferation, and re-patterning. This remarkable capacity stems from the ability of plant cells to make non-committal cell fate decisions (Kidner *et al.*, 2000). During the early stages of root tip regeneration, cells near the wound exhibit mixed or chimeric transcriptional identities, enabling many of them to temporarily function as stem cells and proliferate, as cell division is a crucial step in regeneration (Efroni *et al.*, 2016). Interestingly, some early events in root tip restoration occur independently of cell division, such as the reactivation of the Quiescent Centre (QC)-specific marker, WUSCHEL-RELATED HOMEODOMAIN 5 (WOX5) in the ground tissue above cut end as early as 4 hours post-cut (hpc), even without cell division (Rahni *et al.*, 2024). This early response reflects biochemical changes in cells escaping injury, including the rapid induction of the stress hormone jasmonic acid (JA) within seconds of resection (Glauser *et al.*, 2009; Zhou *et al.*, 2019). Wound induced JA accumulation has been shown to promote stem cell reactivation and regeneration through a gene regulatory network that stimulates the cell cycle regulator CYCD6;1 and the stress-response protein ETHYLENE RESPONSE FACTOR 115 (ERF115) (Zhou *et al.*, 2019). ERF115 plays a crucial role in root tip regeneration (Heyman *et al.*, 2016). Recent studies show that cell wall damage or

modulation of the cell wall components were sufficient to activate the wound induced transcription factor, ERF115 (Hoermayer *et al.*, 2020; Zhang *et al.*, 2022). ERF115 has been shown to act redundantly with its closest homolog ERF114, which can be induced by Brassinosteroid (BR) signalling (Canher *et al.*, 2022). Cell wall damage can activate BR signaling, which in turn modifies cell wall architecture and increases extensibility (Wolf *et al.*, 2012). Thus, the earliest regenerative responses arise through stress-response proteins activated by cell wall damage and mechanical strain during wounding.

Another key player in root tip regeneration is the plant hormone auxin. ERF115 modulates auxin signaling during root tip restoration, and downregulation of ERF115 severely attenuates auxin biosynthesis during this process (Matosevich *et al.*, 2020; Canher *et al.*, 2022). Auxin is not only essential for root tip restoration but also sufficient to trigger it. For example, external application of auxin can induce regeneration in roots cut near the transition zone (high cuts), where regeneration typically fails (Matosevich *et al.*, 2020). Auxin biosynthesis is required at every stage of regeneration, including the induction of transient and mixed cell fates, early cell proliferation, recovery of cell identity, and pattern restoration (Matosevich *et al.*, 2020). Moreover, auxin acts upstream of key regulators of stem cell identity, such as the PLETHORA (PLT) transcription factors (Ding & Friml, 2010).

PLETHORAs (PLTs) are plant specific transcription factors characterized by their AP2 domains. They are key regulators of root development and regeneration, and exhibit gradient expression patterns. PLTs exhibit functional redundancy, with four members—PLT1, PLT2, PLT3, and PLT4—playing crucial roles in maintaining root stem cells. PLTs are highly dose-dependent: very high levels promote stem cell identity and maintenance,

moderately high levels drive the proliferation of stem cell daughters, and low levels lead to differentiation. This dose-dependent activity is governed by a PLT gradient across the root meristem (Galinha *et al.*, 2007). The gradient enables region-specific gene activation, translating into distinct cellular responses (Santuari *et al.*, 2016). Among the gradient expressed PLTs, PLT2 is of particular interest as it has been shown to dictate the boundary for root tip regeneration potential. Roots resected close to the QC (low cuts), where PLT2 levels are high, can regenerate, while roots cut farther proximal (high cuts), where PLT2 levels are low, fail to do so (Durgaprasad *et al.*, 2019). This decline in regeneration potential correlates with changes in gene expression (Efroni *et al.*, 2016). Manipulating the PLT2 gradient prior to cut alters regeneration outcomes: a shortened gradient caused by PLT2 downregulation prevents regeneration even after low cuts, while an expanded gradient enables regeneration after high cuts. This suggests that PLT2 dosage conditions cells before injury, making them susceptible to the biochemical and mechanical changes necessary for regeneration. Although it is clear that PLT2 plays a critical role in conditioning cells for regeneration, the exact mechanisms by which PLT2 dosage influences these biochemical and mechanical changes remain poorly understood.

In this study, I investigated how PLT2 dosage regulates cellular and molecular mechanisms essential for root tip regeneration. I explored how PLT2 conditions cells to become competent for regeneration by altering their transcriptional and physical states before injury. Building on the findings from chapter VII that deformation of cuboidal cells into rhomboid shapes is a prerequisite for successful root restoration, I focused on understanding how PLT2-driven changes in gene expression and cellular geometry

contribute to this process. Through this work, I aim to shed light on the interplay between transcriptional regulation, cell geometry, and regeneration competency, providing a comprehensive framework for understanding the molecular and physical prerequisites for root tip restoration.

VIII. 2. RESULTS

VIII. 2.1. High level of PLT2 is necessary prior to injury for generating the rhomboid cell geometry and ensuring successful root tip restoration

During my investigation of root tip regeneration in *Arabidopsis thaliana*, I observed that PLT2 expression levels are dynamic, consistent with previous findings (Figure 8.1) (Durgaprasad *et al.*, 2019). Given PLT2's critical role in root tip regeneration, I examined the regeneration capacity of the *plt1, plt2* double mutant, as PLETHORA 2 functions redundantly with its homolog, PLT1. The *plt1,plt2* double mutant (Aida *et al.*, 2004) has been shown to be completely impaired in root tip regeneration (Durgaprasad *et al.*, 2019). The length of the root meristem in *plt1,plt2* double mutant is shorter compared to the WILDTYPE (Figure 8.2.A - 8.2.B) (Aida *et al.*, 2004; Durgaprasad *et al.*, 2019). I wondered if the loss of root tip regeneration in the *plt1,plt2* double mutant was due to the relative height of the cuts, as the meristem is shorter in these mutants compared to WILDTYPE. Therefore, I resected the root tips of 4 days post-germination (dpg) *plt1,plt2* double mutant (Columbia-0 background) seedlings at 70µm-100µm and 40µm-70µm from the fourth tier of the columella, and found that these roots did not regenerate at all (Figure 8.2.C - 8.2.E). Next, I asked if the regeneration competency declined with the age of the seedling. Therefore, I

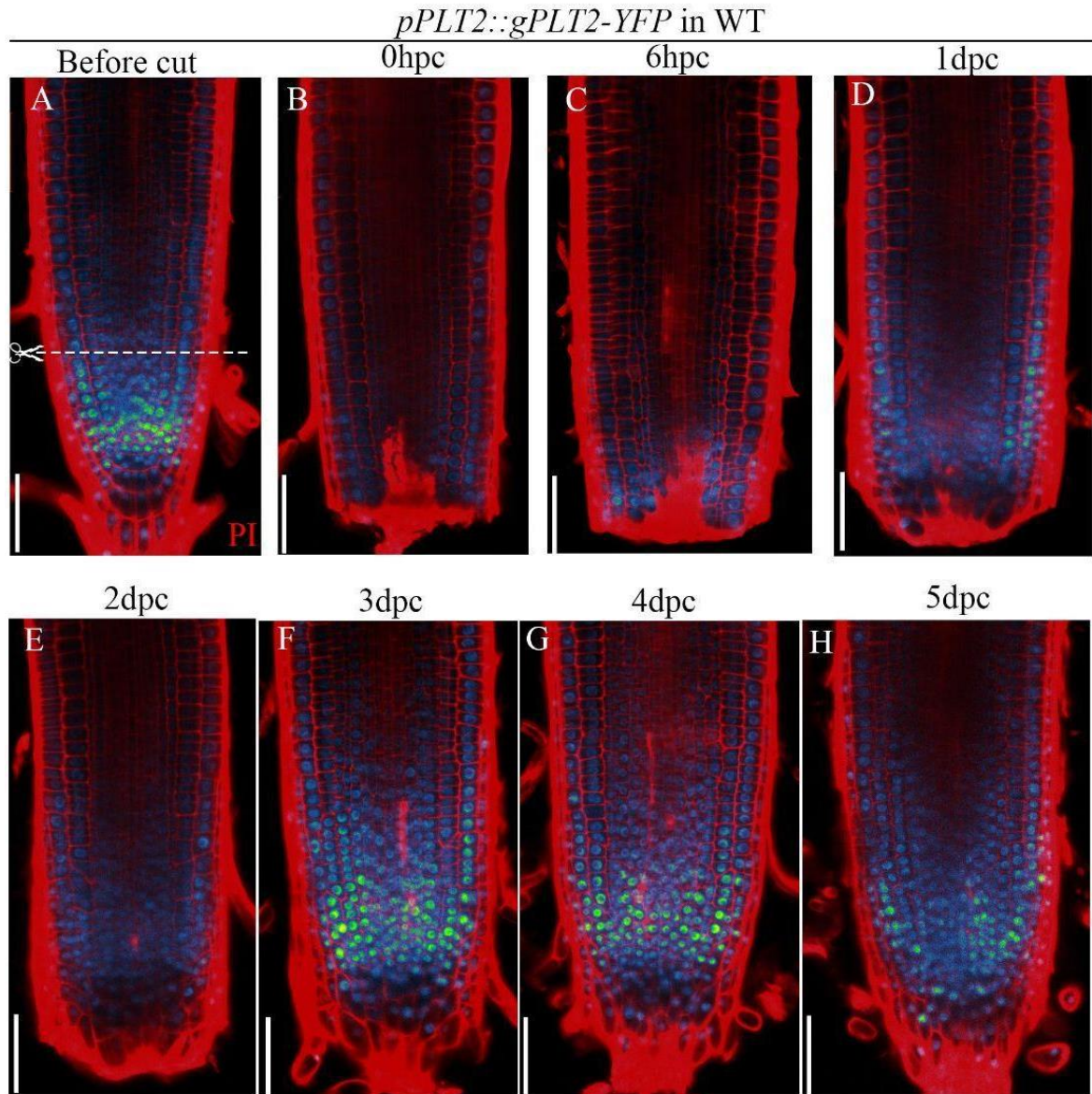


Figure. 8.1: PLT2 expression is dynamic in a regenerating root tip. (A-H) Time lapse imaging of a low cut wildtype root meristem expressing *pPLT2::gPLT2-YFP* undergoing regeneration. The range indicator reports the level of PLT2; blue-low, yellow-high. Red: PI stain. Scale bars: 50µm.

resected the root tips of 2dpg *plt1,plt2* double mutant seedlings at 40µm-70µm (the shortest distance at which the SCN can be removed by resection), and found that the roots still failed

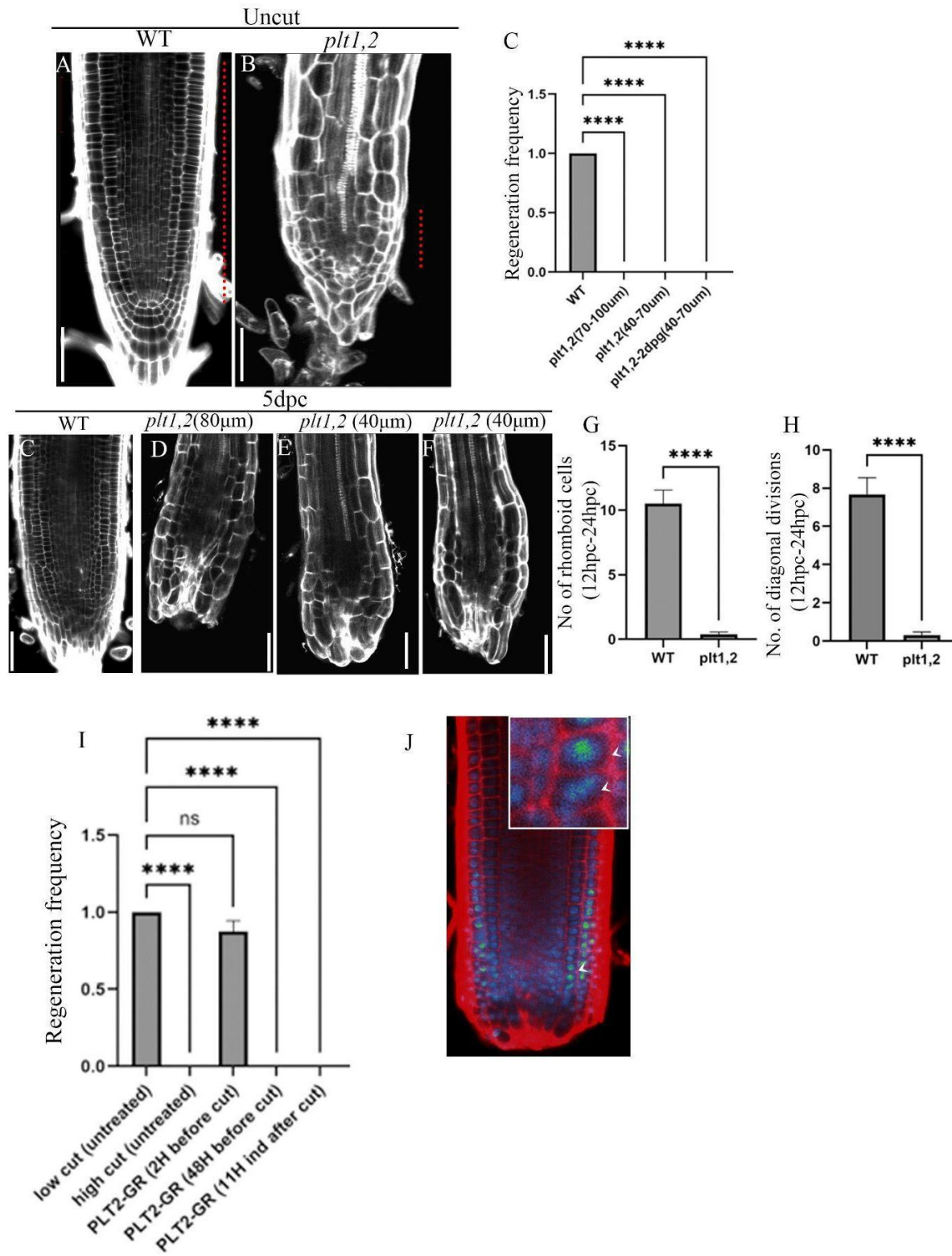


Figure. 8.2: PLT2 is instrumental for restoration of injured root tip. (A-B) Representative confocal images of 4-dpg WILDTYPE (A) and *plt1,2* mutant (B) uncut roots. **(C)** Graphical representation of root regeneration frequency in *plt1,2* mutant compared to wildtype (Kruskal-Wallis test; ****p-value < 0.0001). **(D)** Representative confocal images of wildtype and *plt1,2* mutant 5-days post cut (dpc). **(G)** Graphical representation of number of rhomboid cells per root in wildtype and *plt1,2* mutant at 12hpc-24hpc (Unpaired *t*-test; ****p-value < 0.0001). **(H)** Graphical representation of number of diagonal cell divisions per root in wildtype and *plt1,2* mutant at 12hpc-24hpc (Unpaired *t*-test; ****p-value < 0.0001). **(I)** Graphical representation of root tip restoration frequency upon PLT2 overexpression for 2hours prior to high cut, 48hours prior to high cut and 11hours after high cut (Kruskal-Wallis test; ****p-value < 0.0001 for wildtype low cut v/s wildtype high cut, ns p-value = 0.9426 for wildtype low cut v/s PLT2-GR (2H) followed by high cut, ****p-value < 0.0001 for wildtype low cut v/s PLT2-GR (48H) followed by high cut, ****p-value < 0.0001 for wildtype low cut v/s high cut followed by PLT2-GR (11H)). **(J)** Representative confocal images of a low cut wildtype root expressing PLT2-YFP at 1-dpc. Inset shows rhomboid cells with high PLT2. The range indicator reports the level of PLT2; blue-low, yellow-high. Red: PI stain. Scale bars: 50µm. Error bars: S.E.M. n=sample number

to regenerate (Figure 8.2.C, 8.2.F). This reinforces the previous findings that PLT2 is indispensable for root tip regeneration. Remarkably, the *plt1,plt2* double mutant roots did not generate rhomboid cell geometry after resection nor exhibit a RIGED zone.

Building on the previous findings that PLT2 levels prior to cut are essential for regeneration, I next investigated the role of PLT2 expression in the activation of rhomboid cell geometry during root tip restoration. From previous studies, it was clear that only regions with high PLT2 levels prior to cut were competent to regenerate the root tip (Durgaprasad *et al.*, 2019). Consistent with this, I found that overexpression of PLT2 prior to the cut was effective in promoting root tip restoration, whereas overexpressing PLT2 after the cut did not trigger regeneration, highlighting the necessity of PLT2 levels being high before the resection (Figure 8.3.I). For this experiment, I overexpressed PLT2 under a constitutive promoter using an inducible system in which PLT2 is fused to the Glucocorticoid Receptor

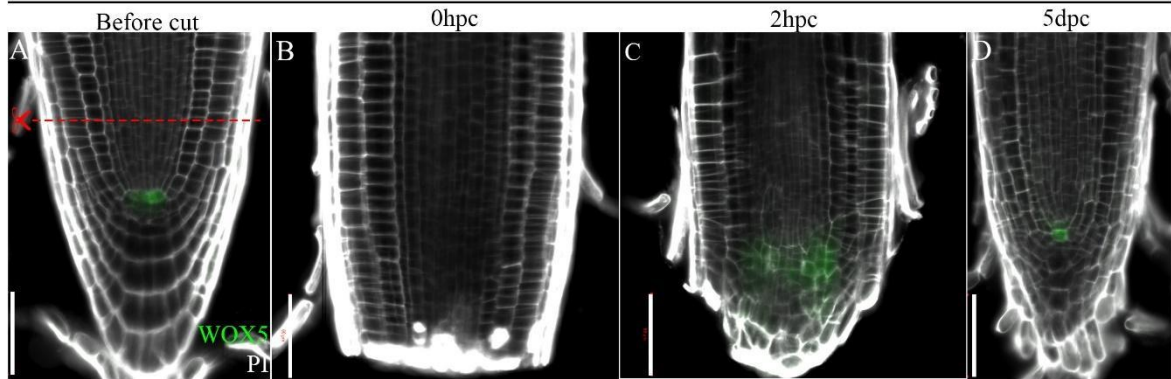
(GR) hormone-binding domain (*35S::PLT2-GR*) (Santuari *et al.*, 2016). Next, I examined whether cells with high PLT2 expression could undergo the cell geometry change to rhomboid shapes. In Chapter 7, I showed that deformation of cuboidal cells into rhomboid shapes and the ensuing diagonal plane of divisions were prerequisites for successful root tip restoration. To investigate this, I performed confocal-based time-lapse imaging on wildtype roots expressing PLT2-YFP (*pPLT2::gPLT2-YFP*). I resected the root within 150µm from the fourth-tier of columella, where PLT2 expression is high. At 24hpc, I observed the activation of rhomboid cell geometry within the RIGED zone (as discussed in Chapter 7), and interestingly, these rhomboid-shaped cells exhibited high levels of PLT2 expression, at least qualitatively (Figure 8.2). As demonstrated in Chapter 7, resecting the root at a higher position—where PLT2 levels are low—failed to induce the formation of rhomboid-shaped cells.

In summary, my findings demonstrate that high levels of the transcription factor PLT2 are crucial prior to resection for both the deformation of cuboidal cells into rhomboid cells and the successful restoration of the root tip. This underscores the importance of PLT2 in priming cells for regeneration.

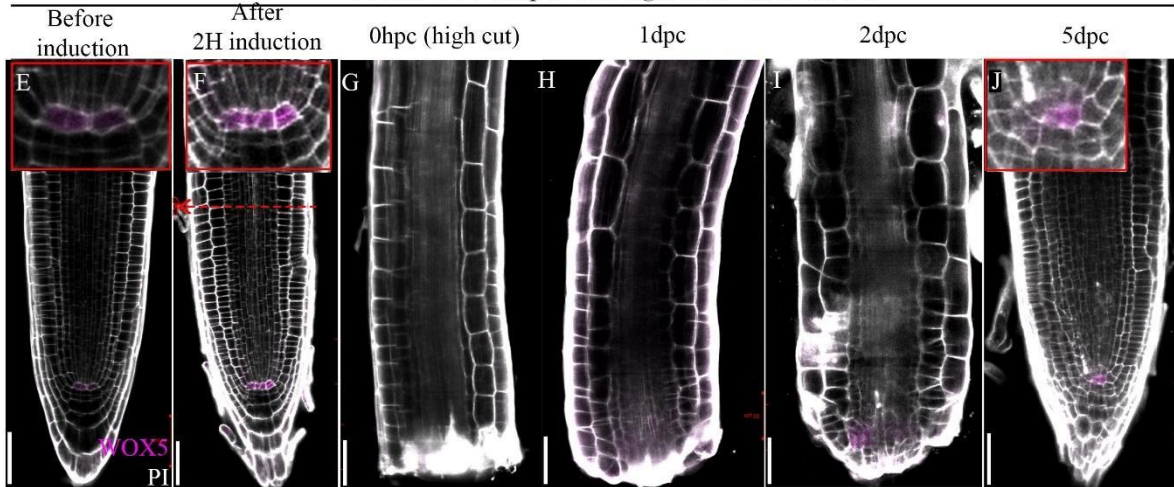
VIII. 2.2. PLT2 levels modulate the expression of the QC marker, WOX5

PLTs are known as master regulators of root development, with varying PLT levels controlling the region-specific induction of genes and feedback mechanisms (Santuari *et al.*, 2016). To explore how overexpression of PLT2 alters the molecular landscape, I investigated the dynamics of WOX5, a homeobox transcription factor crucial for maintaining the root

pWOX5::gWOX5-GFP in WT



35S::PLT2-GR,pWOX5::gWOX5-GFP in WT



35S::PLT2-GR,pWOX5::gWOX5-GFP in WT

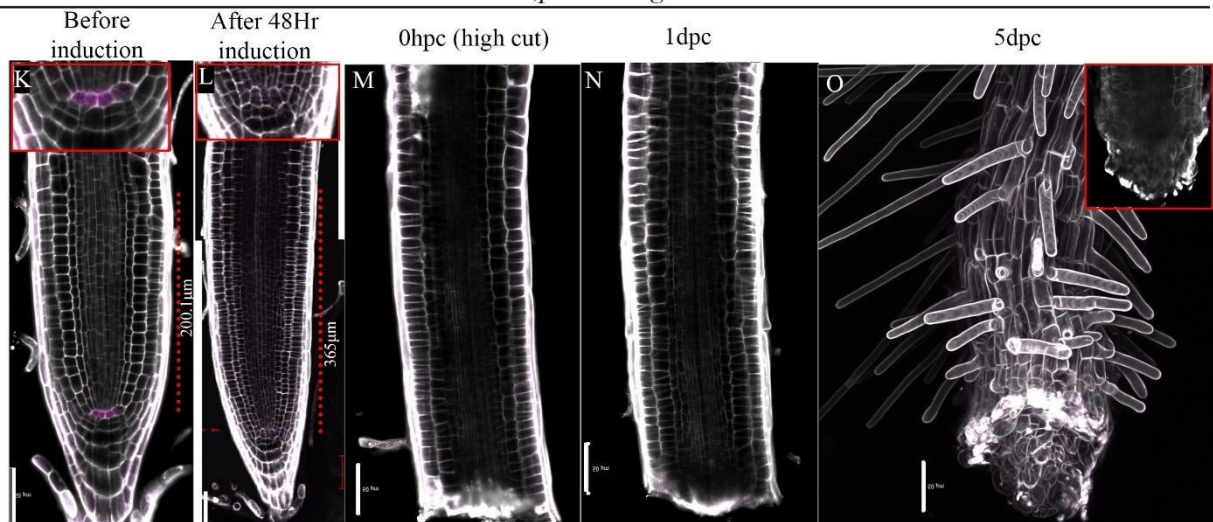


Figure. 8.3: Dosage of PLT2 impact the expression of WOX5 and root tip restoration. (A-B) Live images of a root tip expressing WOX5-GFP (green) undergoing regeneration after low cut . Live imaging of a root tip expressing WOX5-GFP (magenta) following 2 hours of PLT2 overexpression prior to excision, capturing the regeneration process after a high cut. (K-O) Live imaging of a root tip expressing WOX5-GFP (magenta) following 48 hours of PLT2 overexpression prior to excision, capturing the regeneration process after a high cut. Scale bars: 50µm.

quiescent center (QC) in the stem cell niche. Consistent with a previous study (Durgaprasad *et al.*, 2019), I found that after two hours of PLT2 overexpression, the morphology of uncut roots remained similar to untreated wild-type roots, and WOX5 expression in the QC was unaffected (Figure 8.3.A - 8.3.J). It is known that high cuts to roots with PLT2 overexpression for 2 hours prior to cut, can trigger root tip restoration (Durgaprasad *et al.*, 2019). Building on this, I examined WOX5 expression dynamics during PLT2-induced (2 Hours of PLT2 over expression prior to cut) root regeneration upon high cuts. After high cuts, I observed that WOX5 expression returned to the ground tissue above the cut site, forming a broad domain at 24 hours post cut (hpc) (Figure 8.3.H). By five days post cut (dpc), WOX5 was confined to the newly restored QC (Figure 8.3.J). Thus, the return of WOX5 during PLT2-induced regeneration upon high cuts, mimicked the expression pattern seen in untreated wild-type roots after low cuts.

Sustained overexpression of PLT2 for 48 hours has been shown to increase meristem length in uncut roots but abolish root tip restoration after injury (Durgaprasad *et al.*, 2019). To explore this, I investigated WOX5 dynamics during the extended PLT2 overexpression (48 hours). I observed something surprising: WOX5 expression disappeared from the quiescent center (QC) (Figure 8.3.J - 8.3.K). Apart from the meristem doubling in length,

the overall root morphology and patterning remained unchanged (Figure 8.3.K). However, when I resected these roots, WOX5 did not reappear, even after one- or five-days post cut (dpc) (Figure 8.3.L - 8.3.M). These roots failed to regenerate, suggesting that sustained PLT2 overexpression significantly alters the molecular landscape of the root meristem, preventing its ability to regenerate (Figure 8.3.N).

In summary, I demonstrate that PLT2 dosage critically regulates WOX5 expression, both during normal root development and in response to root tip regeneration.

VIII. 2.3. PLT2 acts via its downstream targets to generate specific cell geometry and activate root tip restoration

I next explored how PLT2 dosage alters the genetic program of cells, affecting their ability to regenerate. Specifically, I investigated how PLT2-driven changes in the transcriptional landscape influence cell deformation into rhomboid shapes and root tip restoration after resection. To do this, I performed whole transcriptome profiling using RNA sequencing (RNA-seq). For the experiment, I treated *35S::PLT2-GR* (PLT2-ox) seedlings with dexamethasone (DEX) for 2 hours before making high cuts on their root meristems. At 8 hours post-cut (hpc), I collected approximately 2000–2500 root meristems by excising them below the hypocotyl. Similarly, I collected root meristems from *35S::PLT2-GR* seedlings induced for 48 hours (Figure 8.4). I selected 8hpc as the collection time because PLT2 expression peaks at this time point (Durgaprasad *et al.*, 2019).

I identified 4548 differentially expressed genes (DEGs) between 2 hours and 48 hours of PLT2 overexpression (PLT2-ox), with 2918 genes upregulated (DUGs) and 1630

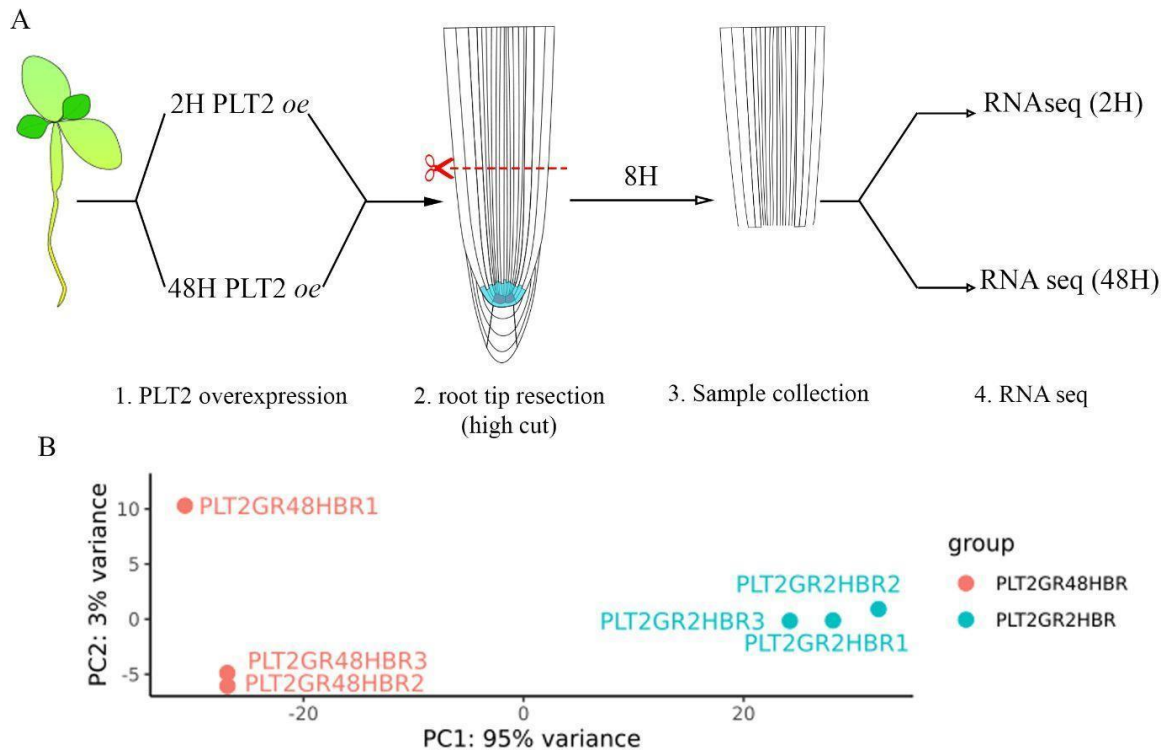


Figure. 8.4: Whole Transcriptome Analysis of resected root tip after 2hours and 48hours of PLT2 over expression. (A) Schematic depiction of the sample collection for RNA-Sequencing. **(B)**Principal component analysis (PCA) plot generated in DESeq2 showing variation within and between groups. Horizontal and vertical axes show two principal components that explain the greatest proportion (95% and 3%) of variation.

genes downregulated (DDGs) at 2 hours of PLT2-*oe*. Gene Ontology (GO) analysis of the DUGs at 2 hours revealed enrichment in biological processes related to hormones, cell wall remodeling, and cytoskeletal elements (Figure 8.5). From these categories, I performed a large-scale genetic screen with several enriched genes based on their previously reported roles on root development and cell shape regulation, and analyzed their corresponding single loss-of-function mutants. These included: *an3*, *phb* (*phabulosa*), *exp-8* (*expansin-8*), *hsl-2*, *expa-10* (*expansin a-10*), *fez*, *rgf-3* (*root meristem growth facto-3*), *bs-1*

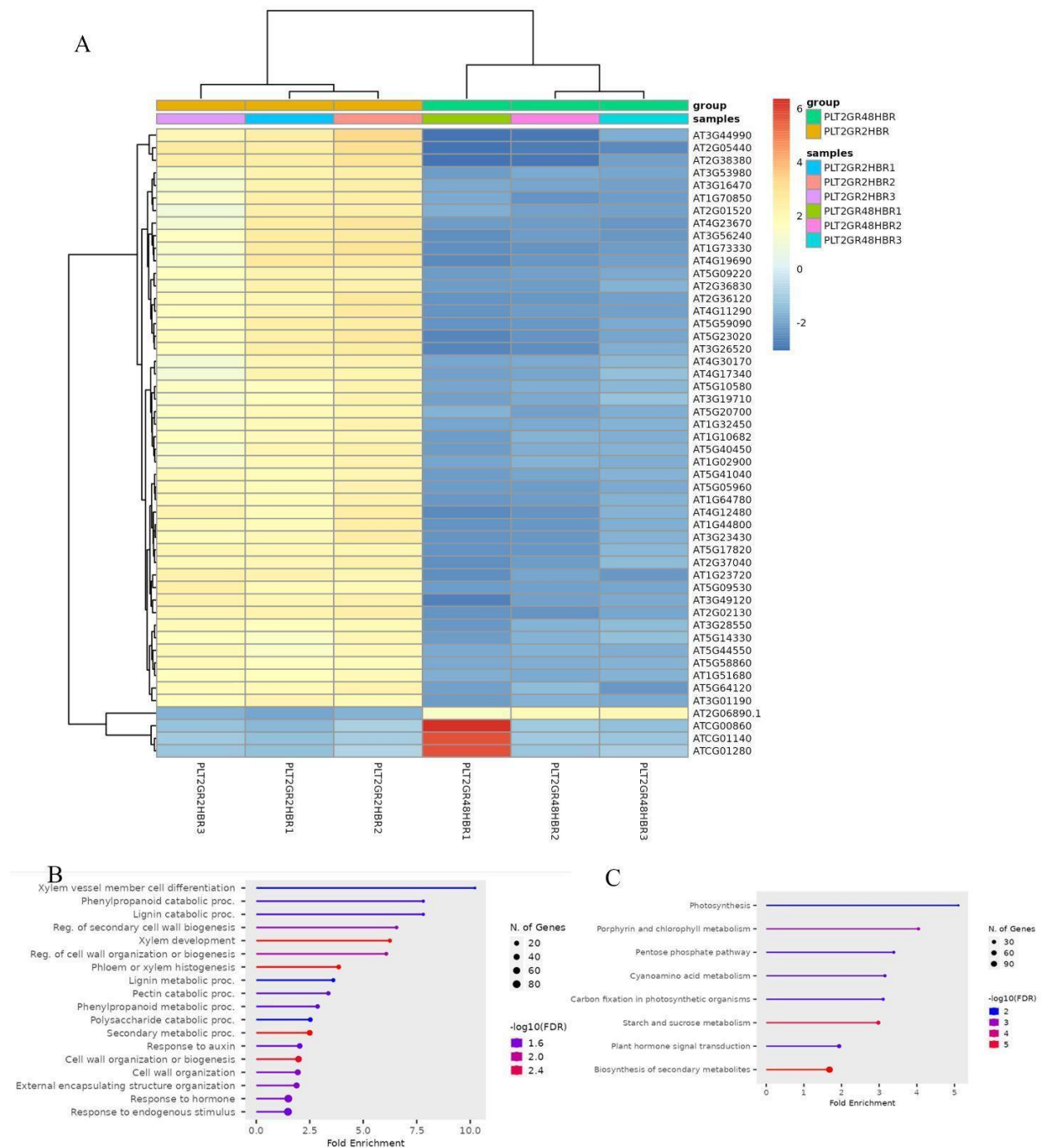


Figure. 8.5: levels of PLT2 modulate the transcriptome. (A) Heatmap of regularised log transformed values showing top 50 genes with the highest variance across samples. **(B)** DUGs in resected root tips when PLT2 was overexpressed for 2hours prior to high cut. **(C)** DDGs in resected root tips when PLT2 was overexpressed for 2hours prior to high cut.

(*bri1*(brassinosteroid-insensitive 1)-suppressor 1), *iaa-30* (indole-3-acetic acid inducible 30) and *expb-1* (expansin b1). Despite their mutations, these single mutants successfully regenerated and restored the root tip after low cuts (Figure 8.6.A). Since cell wall remodelers were enriched, I investigated the role of a specific cell wall-modifying enzyme, PECTIN METHYL ESTERASE INHIBITOR 5 (PMEi5). PMEi5 belongs to the subfamily of PMEIs known to counteract PME activity, reducing cell wall elasticity and increasing stiffness (Peaucelle *et al.*, 2008, 2011). I speculated that overexpression of PMEi5 would impair rhomboid cell formation and disrupt root tip restoration. Preliminary results from a constitutive PMEi5 overexpression (PMEi5-ox; from Prof. Joris Sprakel, WUR, Netherlands) genetic background supports this hypothesis. Further analysis of this genetic background is ongoing in the lab.

As a quick proof of concept, I investigated the role of cytokinins, as Arabidopsis response regulators (ARRs) that play a key role in cytokinin signaling in plants were enriched among the DDGs. I treated resected wildtype seedling roots with 1 μ M trans-zeatin (TZ), a naturally occurring cytokinin derivative, immediately after resection. By 2 days post-cut (2dpc), WOX5 expression had diminished, indicating changes in the molecular landscape (Figure 8.6.B - 8.6.E). Additionally, treatment with 1 μ M or 5 μ M TZ significantly reduced the number of rhomboid cells and diagonal cell divisions compared to untreated controls (Figure 8.6.F - 8.6.H). Ultimately, TZ-treated roots

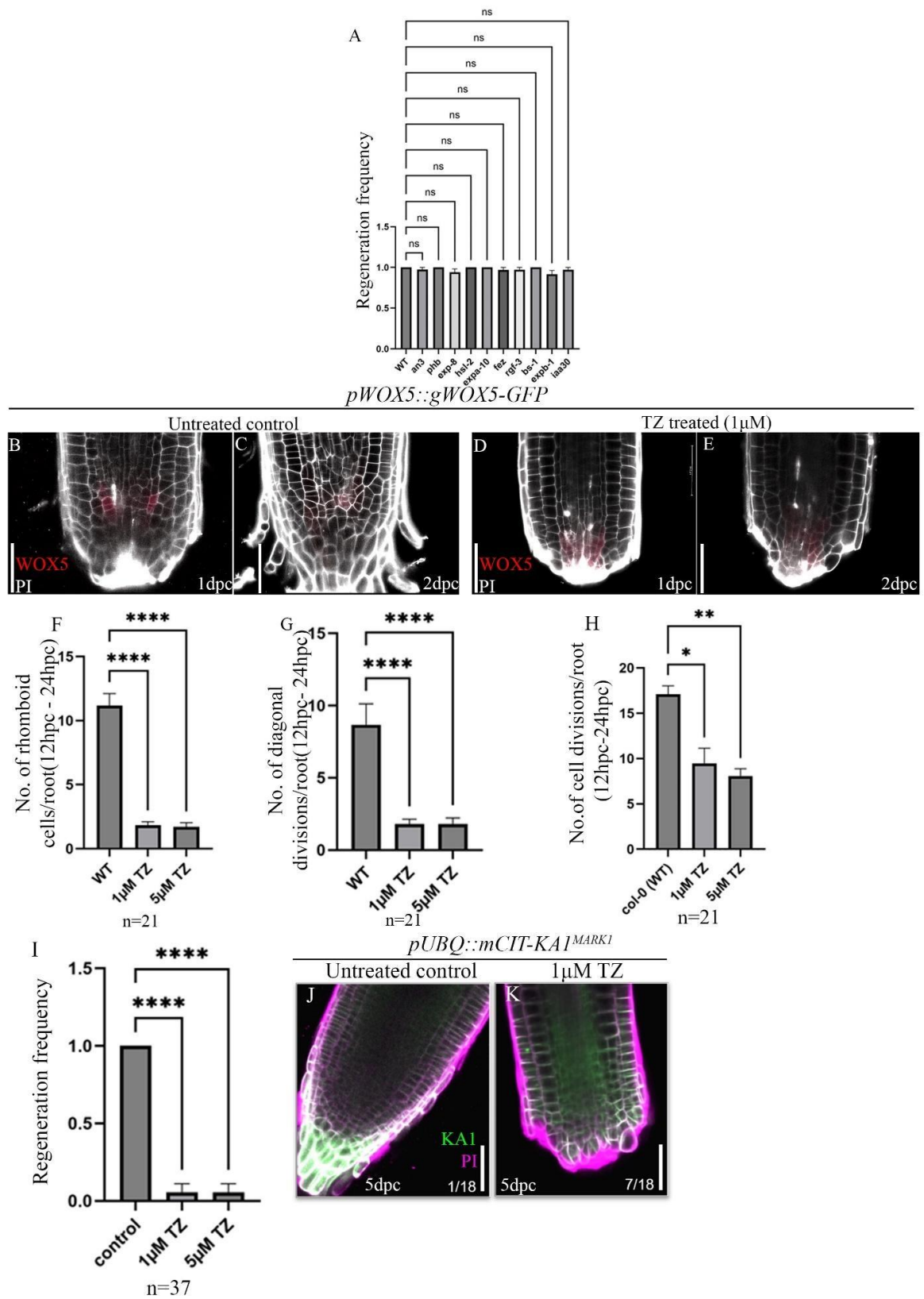


Figure. 8.6: Excess of cytokinin impairs the generation of specific cell geometry and activation of root tip restoration. (A) Graphical representation of root tip restoration frequency in the single mutants screened from the RNA-seq data (n=32, Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, p-value = ns). (B-E) WOX5 (red) expression remain unperturbed and comparable to control even after incubation with 1 μ M transzeatin (TZ) from 0hpc- 48hpc (hour post cut). (F) Graphical representation of number of rhomboid cells observed at 10hpc-24hpc per root when treated for 24hours (0hpc-24hpc) with 1 μ M TZ and 5 μ M of TZ treatment from 0hpc-24hpc (Ordinary one-way Anova; n=21; ****p-value < 0.0001 between wildtype (control) v/s 1 μ M TZ, n=21; ****p-value < 0.0001 between wildtype (control) v/s 5 μ M TZ. (G) Graphical representation of number of diagonal cell divisions observed at 10hpc-24hpc per root when treated for 24hours (0hpc-24hpc) with 1 μ M TZ and 5 μ M of TZ treatment from 0hpc-24hpc (Ordinary one-way Anova; n=21; ****p-value < 0.0001 between WILDTYPE (control) v/s 1 μ M TZ, n=21; ****p-value < 0.0001 between wildtype (control) v/s 5 μ M TZ. (H) Graphical representation of number of cell divisions observed at 10hpc-24hpc per root when treated for 24hours (0hpc-24hpc) with 1 μ M TZ and 5 μ M of TZ treatment from 0hpc-24hpc (Ordinary one-way Anova; n=21; *p-value = 0.0102 between wildtype (control) v/s 1 μ M TZ, n=21; **p-value = 0.0026 between wildtype (control) v/s 5 μ M TZ. (I) Graphical representation of root tip restoration frequency when treated for 5days (0hpc-5dpc) with 1 μ M TZ and 5 μ M of TZ treatment from 0hpc-24hpc (Ordinary one-way Anova; n=37; *p-value = 0.0102 between wildtype (control) v/s 1 μ M TZ, n=37; **p-value = 0.0026 between wildtype (control) v/s 5 μ M TZ. (J) Representative live images of untreated and 1 μ M TZ treated roots at 5dpc. Scale bars: 50 μ m. Error bars: S.E.M. n=sample number

failed to restore the root tip, highlighting the critical role of cytokinin regulation in regeneration (Figure 8.6.I - 8.6.K).

In summary, these results show that PLT2 dosage modulates the transcriptional landscape of root meristems, influencing hormonal responses, cell wall dynamics, and cytoskeletal organization. Perturbations in these processes, such as increased cytokinin activity, disrupt rhomboid cell formation, ultimately impairing root tip regeneration.

VIII. 3. DISCUSSION

In this chapter, I investigated the dosage-dependent role of PLT2 in modulating cellular competence for root tip regeneration in *Arabidopsis thaliana*. I demonstrated that high levels of PLT2 prior to injury is essential for successful generation of rhomboid cells geometry and root tip restoration, with its overexpression post-injury being insufficient. PLT2 influences the molecular and cellular landscape by regulating key factors such as WOX5 expression, root tip restoration. Through RNA sequencing, I identified transcriptional changes driven by PLT2, including enrichment of genes involved in hormonal signaling, cytoskeletal organization, and cell wall remodeling. Notably, perturbation of cytokinin levels disrupted rhomboid cell geometry, WOX5 dynamics, and regenerative ability, emphasizing the importance of a finely tuned molecular landscape for regeneration.

These findings highlight the intricate interplay between PLT2 dosage, cellular geometry, and the transcriptional landscape in enabling root tip regeneration. A key hypothesis emerging from this work is that PLT2 establishes a biochemical and biophysical state within the root meristem, priming cells for rhomboid geometry formation and restoring WOX5 expression to the regenerated quiescent center. This state may involve precise modulation of hormonal responses, such as cytokinin activity, and mechanical properties, such as cell wall elasticity, to orchestrate regeneration. Alterations to this balance, whether through sustained PLT2 overexpression or external induction of its downstream targets, result in a disrupted regenerative process, suggesting that PLT2 acts as a master regulator integrating multiple pathways to ensure successful tissue restoration. Although my current

study focuses on PLT2 levels prior to the cut, I do not discount the importance of PLT2 throughout the regeneration process. The dynamic expression of PLT2 during regeneration may transiently alter cell behavior, enabling timely adjustments in gene expression and cell mechanics essential for initiating regeneration.

Future studies should focus on dissecting the downstream targets and pathways regulated by PLT2 in greater detail. For instance, functional validation of candidate genes identified in the transcriptome analysis, including those involved in hormonal signaling and cell wall remodeling, will provide deeper insights into their roles in regeneration. Advanced imaging techniques and biophysical assays could elucidate how PLT2-mediated changes in cell wall mechanics and cytoskeletal dynamics influence rhomboid cell formation.

Chapter IX: Conclusion

This work is a part of the published manuscript:

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([†] *Co-corresponding author*)

IX. Conclusion

The development and morphogenesis of organisms have captivated scientists for centuries. Early interpretations of these processes were rooted in the concept of morphogens. Morphogens biochemical signals that guide tissue organization and patterning by forming spatial concentration gradients. These gradients regulate gene expression and cell behavior, laying the foundation for developmental precision. In 1952, Alan Turing advanced this understanding by proposing a theory of morphogenesis based on the reaction-diffusion kinetics of morphogens (Turing, 1952). Even to this day Turing's model is used to explain development and patterning (Robinson & Roeder, 2015; Ding *et al.*, 2020). This continued relevance is based on the role of spatiotemporal regulation of genes in establishing the timing and positioning of cell fate decisions during development and morphogenesis (Nüsslein-Volhard & Wieschaus, 1980; Gilmour *et al.*, 2017). A compelling example is the molecular basis of shoot apical meristem (SAM) development in plants. In the SAM, signaling molecules—including miRNAs, peptides, and hormones—regulate the spatial and temporal expression of transcription factors critical for cell fate (Ha *et al.*, 2010; Sablowski, 2010). The spatiotemporal expression patterns of these biomolecules in a developing SAM are pre-programmed during embryogenesis (Figure 9.1). But, an experimentally amenable system where the expression profile of the biomolecules is not predetermined would open new avenues and fresh insights into development and self-organized morphogenesis. Tissue culture-mediated *de novo* shoot regeneration provides such a system. In this context, cytokinin-rich media trigger the initiation of shoot initials called progenitors. Progenitors

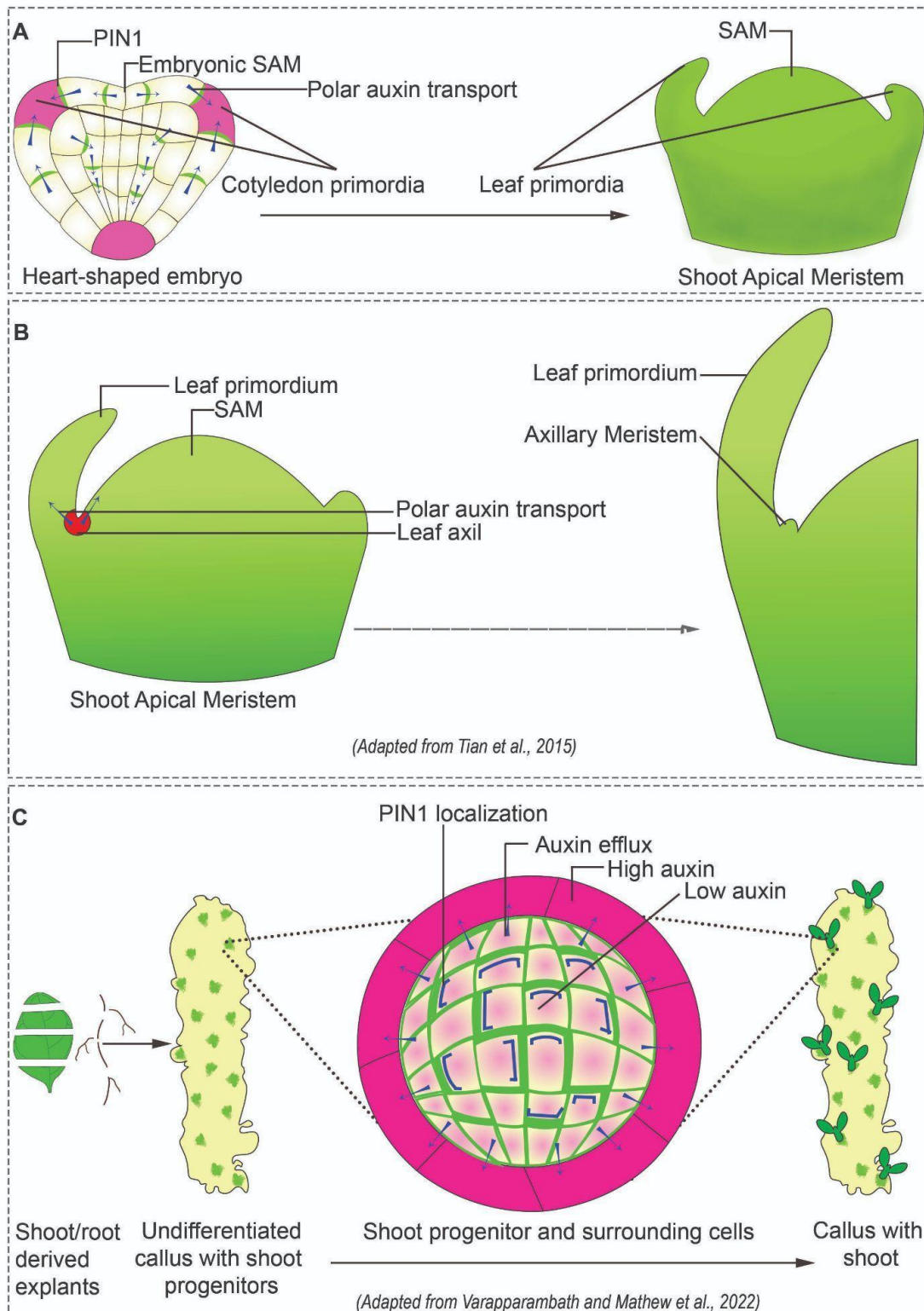


Figure. 9.1: Shoot meristem formation during development and *de novo* regeneration.

(A) During embryogenesis, the cotyledon primordia provide a positional cue and draw in auxin from the embryonic shoot apical meristem (SAM) to generate low auxin conducive for SAM development. (B) During axillary meristem formation, the subtending leaf provides positional cue and draws auxin from its axil to create a low auxin environment. Adapted from Tian *et al.*, 2015. (C) Unlike normal development, *de novo* shoot regeneration from undifferentiated callus via artificial culturing of shoot or root explants occur in the absence of positional cues. The PIN1 in the cells of a regenerating shoot progenitor gets asymmetrically localized onto the plasma membrane, and the cells further self-organize to flush out auxin from the progenitor. This results in two domains of different auxin levels: the progenitor with relatively low auxin levels which progress into shoot meristem, and the surrounding cells with high auxin levels. Blue arrows: Direction of auxin flow (A, B, C). Blue bracket: PIN1 localization pattern (C).

are clusters of PIN1-marked cells that emerge within stochastically formed morphogenic fields. These morphogenic fields are characterized by random groups of cells exhibiting high proliferation (Figure 9.1.C).

I observed that PIN1 and intrinsic cell polarity proteins, such as SOK2, exhibit asymmetric localization on the membranes of progenitor cells. Disruption of this localization hinders the progression of progenitors into the shoot meristem. This strict localization pattern of the auxin efflux carrier, PIN1 likely ensures low auxin levels in progenitors, creating conducive conditions for maintaining shoot stem cell regulators (Clark *et al.*, 1996; Long *et al.*, 1996; Laux *et al.*, 1996; Gordon *et al.*, 2007; Landrein *et al.*, 2015; Zhang *et al.*, 2017; Ma *et al.*, 2019; Varapparambath *et al.*, 2022). This observation raises an intriguing question: how does the system sustain the required pattern of PIN1 localization?

Through my investigations, I realized that this question cannot be addressed solely from a biochemical perspective; it also requires a mechanical viewpoint. This is because the progenitor cells are compressed within a shell of surrounding cells, and the rapid proliferation of progenitor stretches this outer shell. The cell wall-loosening enzyme XTH9, active exclusively in the shell, facilitates its stretching, allowing it to accommodate the

growing progenitors. In essence, the outer shell of cells acts like a rubber shell, compressing its contents into a tight arrangement while also yielding to the growth of their contents by stretching accordingly. This stretching, in turn, stimulates further growth of the progenitors, creating a feedback loop. The balance of forces between compression in the progenitors and tension in the shell maintains the precise PIN1 localization pattern essential for progenitor progression. Even the slightest imbalances in these forces compromises PIN1 localization, ultimately leading to the abortion of the progenitors. A mechanical conflict arises at the boundary where the compressed progenitor cells meet the tensed non-progenitor cells of the outer shell. This conflict causes the progenitor to buckle and, ultimately, protrude out of the shell, forming a dome-shaped meristem. This "buckling" likely arises from a symmetry-breaking event within the progenitor, where the upper layers experience more planar growth compared to the lower layers. This disparity in growth generates mechanical inconsistencies that lead to feedback interactions between neighboring cells. Such feedback is not coincidental; it is likely crucial for preserving the local biochemical environment and ensuring the proper tissue and cell shapes in and around the progenitors. These findings highlight the critical interplay between biochemical signals and mechanical forces in morphogenesis.

The integration of mechanics into development and morphogenesis is not a new concept. In fact, it was first proposed over a century ago by D'Arcy Thompson in 1917, pioneering a new way of thinking for developmental biologists (Thomson, 1917). It quickly became apparent that morphogenesis can only be fully understood by addressing the system in an integrative manner—considering the combination of genetic determinants, molecular

entities, cellular interactions, and the physical environment (Trinkaus, 1984). This integrated approach now allows for a deeper understanding of morphogenesis. It offers insights not only into normal development and tissue culture-mediated organogenesis but also into the formation of specific organ parts, such as the regeneration of the root tip in plants.

In this context, I used root tip regeneration in *Arabidopsis* as a model to explore the shape-forming mechanisms. Through my investigations, I found that the shape of a normally growing root consists of three elemental shapes: a 'cylinder' that gradually narrows into a 'cone frustum,' capped by a rounded cone (or hemisphere). During root tip resection, the regions shaped like the rounded cone and cone frustum are removed, leaving behind only the cylindrical portion. Complete regeneration of the root tip requires the restoration of these lost shapes. I discovered that this restoration occurs in two steps: first, the cylinder is capped by a hemisphere, and second, a cone frustum forms between the cylinder and the hemisphere, thereby fully restoring the lost shapes.

The appearance of the hemisphere in the first step is triggered by increased growth in the stele following resection. This accelerated stele growth causes the cut end, forming the base of the cylinder, to bulge outward in a convex fashion. I identified a critical time window within the duration of this process. During this window, the cell near the cut end, in particular the cortical cells present at the time of the cut undergo geometric deformation, changing from cuboidal to rhomboid. This cell deformation results from the unequal growth between the stele and the epidermis: the inner side of the cortical cells is pulled by the rapidly growing stele, while the outer side remains relatively stagnant, as the epidermis grows only slowly. These rhomboid-shaped cells undergo a unique diagonal division along their shortest

axis, serving as a geometric cue that is key to triggering the second step. The daughter cells from this diagonal division guide the vertically growing cell files to subtly change direction and grow along an inclined trajectory. This marks the onset of tapering, where the cylindrical root begins to narrow into a cone frustum. It is plausible that tapering triggers the deformation of additional cells into a rhomboid shape for a brief period. However, this process is not sustained. As tapering progresses, the appearance of rhomboid cells becomes less frequent and eventually undetectable once the root morphology is fully restored. In summary, I discovered a transient cell geometric cue that orchestrates the shape formation process during root tip regeneration.

Understanding the mechanisms that drive cell self-organization and shape generation was identified as one of the major challenges of the millennium. Despite this, relatively little is known about these processes, with most research focusing on cell division and differentiation. Through my investigations using *de novo* shoot regeneration and root tip regeneration as models, I discovered new mechanisms of shape generation, offering fresh insights into developmental biology. At the heart of my work lies the interplay between biochemistry, mechanics, and cell geometry, in self-organized morphogenesis.

Looking ahead, my discovery lays the groundwork for a more comprehensive understanding of how intrinsic cellular mechanics and geometry contribute to the self-organization of tissues during development. Insights from my studies could reveal previously unexplored principles of morphogenesis, shedding light on how cells and tissues dynamically respond to environmental cues, adapt to mechanical forces, and maintain developmental trajectories. Such insights could reshape our understanding of the

fundamental processes driving morphogenesis and pattern formation, tissue architecture, and regeneration across diverse biological systems.

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