Investigating the interplay of auxin and *PLETHORA7* during *de novo* root regeneration

A Thesis

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by

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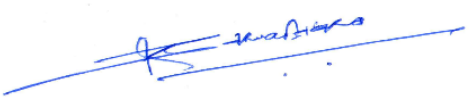
From May 2023 to Mar 2024

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled '**Investigating the interplay of auxin and *PLETHORA7* during *de novo* root regeneration**' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Pratham Shivhare** at Indian Institute of Science Education and Research

under the supervision of **Dr. Kalika Prasad**, Associate Professor, Department of Biology, during the academic year 2022-2023.



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This thesis is dedicated to my Parents
and my brother

Declaration

I hereby declare that the matter embodied in the report entitled '**Investigating the interplay of auxin and *PLETHORA7* in *de novo* root regeneration**' are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of **Dr. Kalika Prasad** and the same has not been submitted elsewhere for any other degree.

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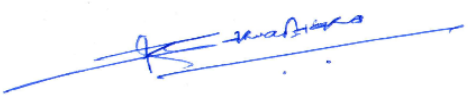

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Abstract

The regenerative abilities of plants range from the individual cells, tissues, and organs to the complete rebuilding of the whole organism. Over the years, *Arabidopsis thaliana,* a model organism, has provided insights into the mechanisms underlying plant regeneration. Regenerative response in *A. thaliana* can be categorized into two basic categories, tissue culture-induced regeneration and mechanical injury-induced regeneration. *De novo* root regeneration (DNRR) is a plant regeneration caused by mechanical injury in which it demonstrated that the mechanical injury-induced regeneration in *A. thaliana* excised leaf generates adventitious roots when in contact with a substrate or wound healing response in the form of a callus when the cut end is kept untouched. Previous research showed that the DNRR response in *A. thaliana* is controlled by transcriptional factors such as PLETHORAs (PLTs). During *de novo* root organogenesis, endogenic auxin plays a critical role to achieve a fate transition of regeneration- competent cells to become the root founder cells. However, it is not well understood how the PLETHORA 7 and auxin response pathways interact and affect each other during *de novo* root regeneration. Therefore, the main aim of this project is to uncover the interplay of the auxin and PLT7 in *A. thaliana* during regenerative response.
The results indicate a significant increase in the expression level of PLT7 upon inhibition of auxin signaling. However, the *de novo* root regenerative assay conducted on the transgenic line (pG1090:XVE::*axr3*-1-RFP) revealed a complete abolishment of *de novo* root regeneration following a 15-minute of estradiol induction. Utilizing double marker lines, we examined the expression pattern of PLT7 in the *axr3*-1 with 15 minutes of estradiol induction and observed that despite the increase in PLT7 expression level, it was not localized near the injury sites but rather distally from the petiole section. The expression pattern of PLT7 in the context of auxin signaling inhibition differed from the normal PLT7 expression pattern observed in regenerative perspectives during *de novo* root regeneration (DNRR). Thus, indicating proper spatiotemporal activity of PLT7 is critical to initiate DNRR not necessarily just the global expression itself.

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Contributions

Contributor name	Contributor role
PS, KP, AG	Conceptualization Ideas
PS, AG	Methodology
PS	Software
PS	Validation
PS	Formal analysis
PS	Investigation
KP	Resources
PS	Data Curation
PS	Writing - original draft preparation
PS	Writing - review and editing
PS	Visualization
KP	Supervision
KP	Project administration
KP	Funding acquisition

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This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Chapter 1 Introduction

Regeneration is a fundamental biological process where organisms replace damaged or lost cells, tissues, or body parts, crucial for survival in different species. It is a fundamental living of many organisms found across various domains of life. All the living being possess some ability to regenerate to some degree produce new cells, tissue or even entire body plans from small fragment of themselves. These are important action for the organisms as they help to maintain their functionality. Animal and plants are highly susceptible to the stress and injuries caused by the various biotic and abiotic factors. To sustain their survival, they have to deal with the stress and injuries. In animal kingdom, we can see different regeneration abilities across various taxa from simple to complex organisms (Poss, 2010), regenerative capacity varies widely due to factors like development and environment. In animals, species like planarians and salamanders demonstrate impressive regenerative abilities due to specialized stem cells that replace lost tissues (King and Newmark, 2012). Planarians can regenerate whole organisms, including heads and organs, through a process called morphallaxis. Similarly, hydras, part of Cnidaria, can regrow from small tissue fragments due to specialized stem cells. These instances showcase diverse regenerative abilities in animals, emphasizing the value of studying model organisms for understanding biological principles.

Although animal regeneration has received much attention, plants also possess impressive regenerative abilities through different mechanisms. Plants show a robust ability to regenerate despite being surrounded by rigid cell walls and cells lacking migration to the site of injury and adaptability in response to damage from biotic and abiotic factors. Plants evoke in themselves a multitude forms of regenerative response to counteract the damage caused by the abiotic and biotic stress (Birnbaum and Alvarado, 2008). It can regenerate many parts of their bodies that include roots, stem, shoots, hypocotyls, leaves and so on (Chatfield et al., 2013; Liu et al., 2014; Kareem et al., 2016). This ability showcases the complex interaction of genetic, physiological, and environmental factors in plant regeneration. Furthermore, plants have evolved mechanisms to sense and respond to environmental stresses, allowing them to adjust regenerative responses. Environmental factors like drought, pathogens, and herbivores can activate specific signaling pathways for gene expression related to regeneration. Hormonal signals like auxin and cytokinin are crucial for coordinating plant regeneration processes, regulating cell division, differentiation, and tissue

patterning. By utilizing these mechanisms, plants can quickly regenerate damaged tissues and organs for survival and reproductive success in challenging environments.

1 Understanding Regeneration in plants using Model system *Arabidopsis thaliana*

The regenerative abilities of plants range from the individual cells, tissues, and organs to the complete rebuilding of the whole organism. Over the years, *Arabidopsis thaliana*, a model organism, has provided insights into the mechanisms underlying plant regeneration because of its tiny genome size, short life span, and simplicity of handling.

There are two main classes of regeneration in *A. thaliana* as follows (Mathew and Prasad, 2021):

1.1 Tissue culture-induced regeneration- In this type of regeneration, an explant (an entire piece or organ of the plant) is used to regenerate the organism in vitro. This process is called *de novo* organogenesis. It can be classified further into *de novo* shoot and *de novo* root regeneration (Figure 1).

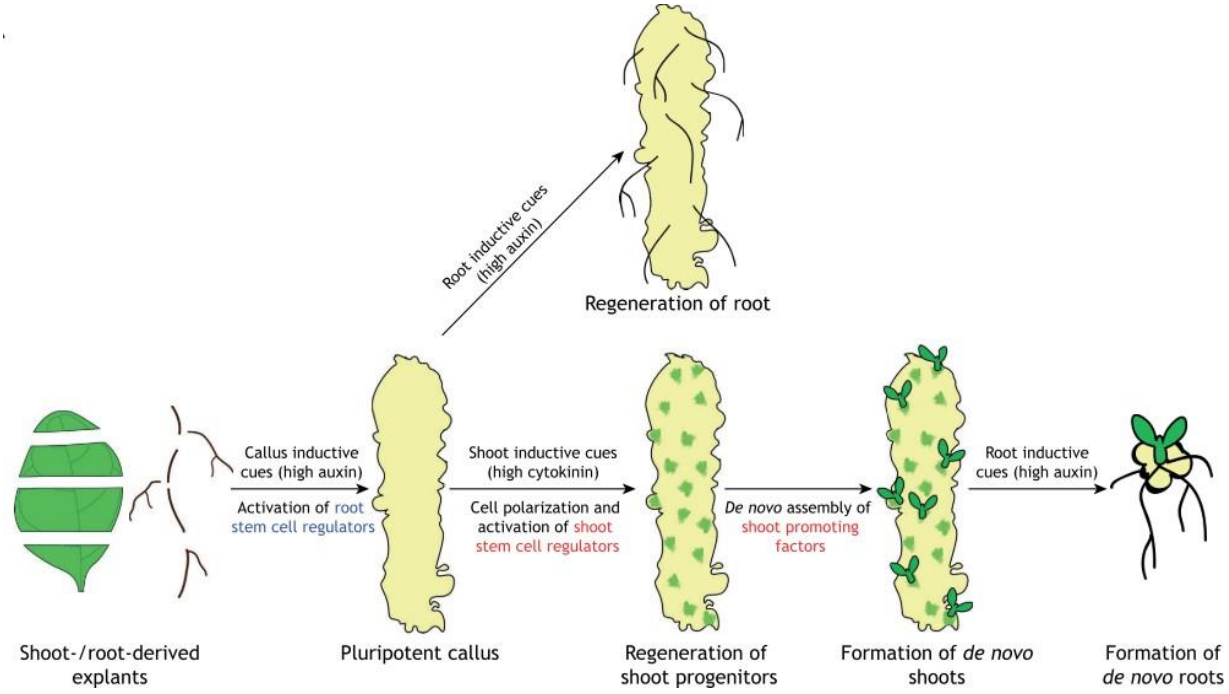

Figure 1: Indirect tissue culture-induced regeneration. Schematic representation of the tissue culture-mediated regeneration, firstly high auxin content provided in the growth media to give rise to pluripotent callus then additional cues provide to give rise to root (top) and shoot (bottom) regeneration. (Adapted From Mathew and Prasad, 2021).

1.2 Mechanical injury-induced regeneration- Plants encounter injuries from both biotic and abiotic factors during their regular growth. Consequently, plants must initiate a regenerative or healing process for the damaged tissues or organs, which triggers diverse mechanical responses across various parts of their bodies. We mimic some of these injuries and check the responses to study and understand the regenerative process (figure 2).

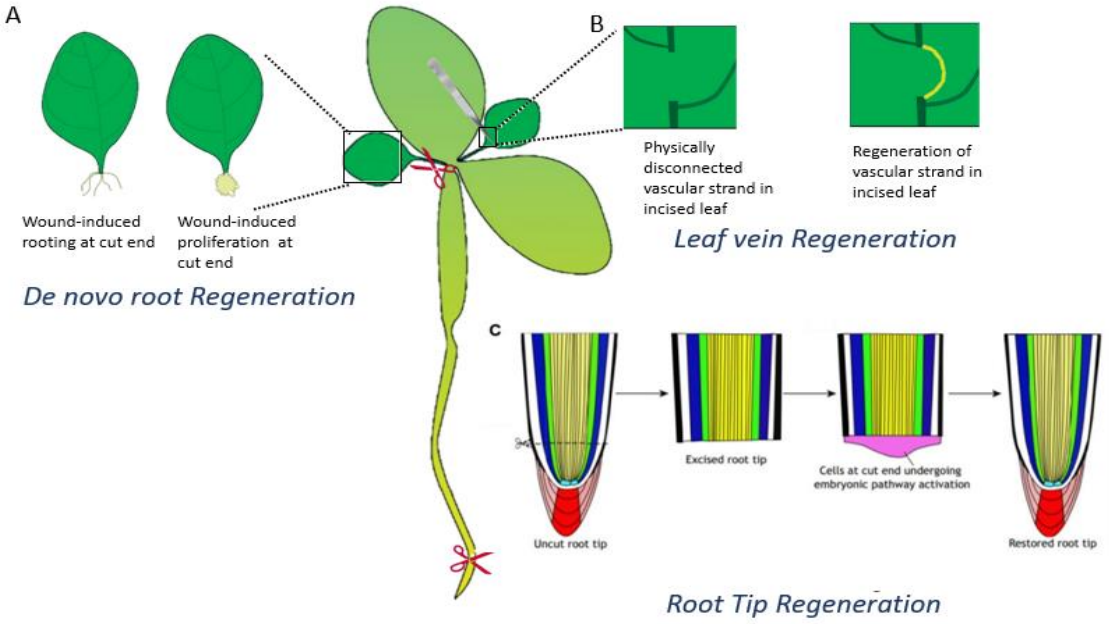

Figure 2: Different types of Mechanical Injury-based regenerative responses in *Arabidopsis*. A. Regeneration response from the cut petiole of a detached leaf when contact to media (root) or not kept with contact to media(callus). B. Vascular regeneration takes place after an incision is made on the leaf mid-vein, and the reestablished vascular stands grow surrounding the injured location then restore vascular continuity. C. Resection of the root tip results in the multiplication of unaffected endodermal cells. This, in turn, leads to a transient activation of embryonic signaling pathways, facilitating the regeneration of the root tip (adapted from Mathew and Prasad, 2021).

1.3 *De novo* root regeneration (DNRR)

when removing an organ or component from the parent plant causes the formation of a new organ with a different identity from the original tissue or organ (i.e.*de novo* organogenesis) (Bustillo-Avendaño et al., 2018).

In *de novo* root regeneration, when a leaf cut or separate from the parent plant it will develop a new root during regeneration interestingly this type of the regeneration is not dependent of any external or exogenous hormones. This type of the regeneration is surely dependent on the endogenous hormones which is mimicking the natural environment condition in which there is no external hormones is available. Previous investigations (Shanmukhan et al., 2021) demonstrated that the mechanical injury-induced regeneration in *A. thaliana* excised leaf generates adventitious roots when in contact with a substrate or a callus when the cut end is kept untouched. The author showed that the physical contact at the cut end with any physical substrate determines the formation of adventitious roots over formation of callus.

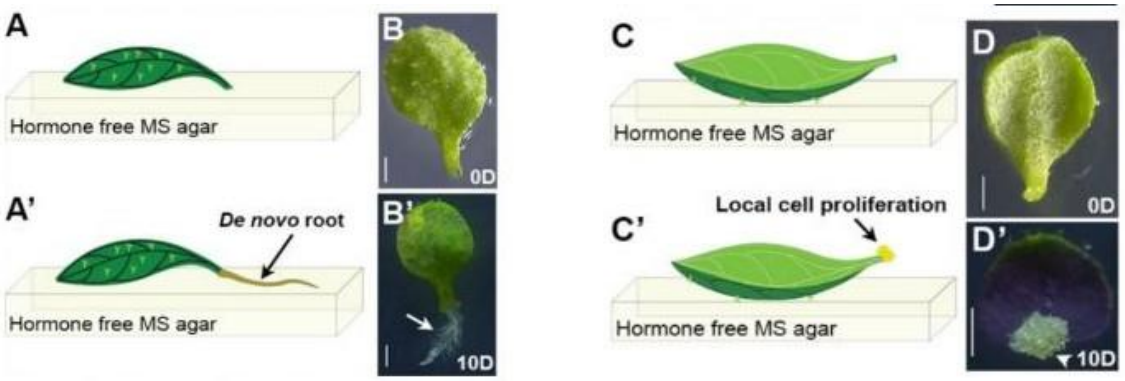

Figure 3: Regenerative response in the *A. thaliana*. *de novo* root regeneration at the cut end of a detached leaf in the presence of touch: (A, A', B, B'). II) Formation of callus when detached leaf placed adaxial side down on the MS-agar media in the absence of touch: (C, C', D, D'). (adapted from Shanmukhan et al., 2021)

To further understand the mechanism behind this DNRR, the researchers developed a unique approach for mimicking natural environmental conditions in order to produce roots from *Arabidopsis thaliana's* numerous organs (Chen *et al.*, 2014; Liu*et al.*, 2014). The detached plant organ must rely on endogenous hormones in this circumstance, which is analogous to the natural scenario in which no external hormones are available.

1.3.1 Framework of DNRR

A cell must undergo reprogramming to adapt to a new organ's identity during the *de novo* organogenesis process. Initially, cells capable of regeneration must dedifferentiate into stem cell-like cells before transforming into cells with a different identity (da Rocha Correa et al., 2012; Chen et al., 2016). Adult stem cells from vascular tissue play a crucial role in promoting new root growth. Proliferation is necessary for organogenesis after cellular reprogramming. Hormone responses and specific gene regulation are key factors in acquiring a new cell fate. Auxin and cytokinin play a crucial role in determining root or shoot destiny during regeneration. High auxin to cytokinin ratio leads to root formation, while high cytokinin to auxin ratio results in shoot formation (Ikeuchi et al., 2019). Auxin is essential for Direct Root Regeneration Response (DNRR) (Pop et al., 2011; Xu, 2018; Mathew and Prasad, 2021). Inhibitors of auxin biosynthesis or transport prevent root regeneration (Pop et al., 2011; Xu, 2018; Mathew and Prasad, 2021).

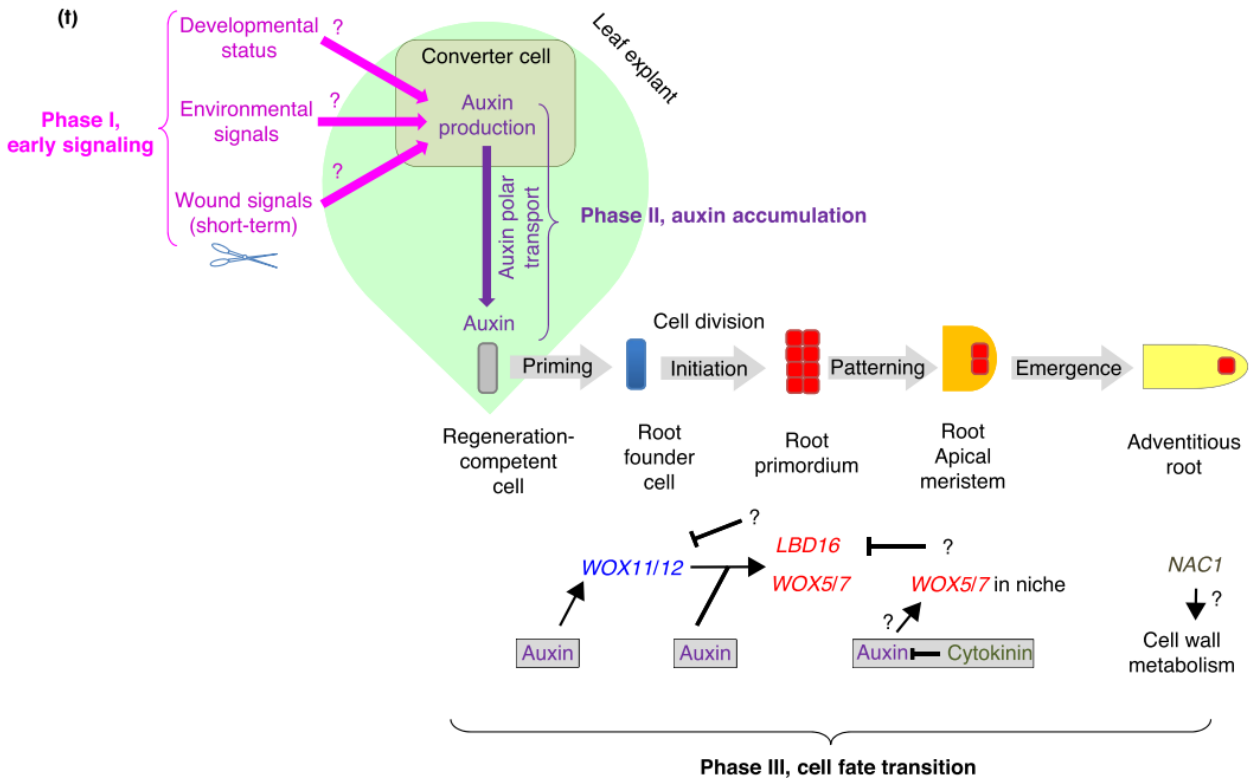

Figure 4: Cellular and Molecular framework of DNRR. Adapted from (Xu, 2018)

Auxin accumulations induced several transcription factors and genes such as ARFs, YUCCA, WOX proteins to help cells acquire new fate (Liu et al., 2014; Chen et al., 2016). Therefore, auxin transport and biosynthesis are vital for DNRR response. In 2018, Lin Xu explained the cellular and molecular framework of the DNRR in three stages. The initial phase involves early signaling through wound detection by explants (Xu, 2018), which receive various environmental and wound cues. Following this, the second phase sees auxin

accumulation in cambium and procambium cells, leading to root formation. In the last phase, cell fate changes occur in four stages, including the conversion of cells into root founders and the emergence of the root tip from the meristem cell layer.

1.4 Leaf vein regeneration

The study conducted by (Radhakrishnan et al., 2021), particularly leaf vascular repair, has profound consequences for plant biology and agriculture. Plant organs, particularly aerial ones such as leaves, are constantly injured by both biotic and abiotic causes. If left untreated, such damage can stifle plant development, weaken immunity, and even risk survival. Therefore, it is critical to understand how plants repair and regenerate injured tissues, particularly their vasculature.

In this leaf vein (pun intended), the author proposed a novel strategy to studying leaf vascular regeneration, a topic that has received less attention compared to other regenerative processes in plants. The authors present a detailed procedure for doing a leaf vascular regeneration experiment, which entails carefully incising the leaf midvein and then observing the regeneration process with micro-surgical techniques and brightfield microscopy. The researchers hope to identify the fundamental elements determining effective vascular regeneration by carefully controlling variables such as leaf age, injury size, and position along the leaf blade.

One of the study's primary findings is that injury size has a vital influence in influencing regeneration outcomes and found that smaller incisions (less than 400 µm) were more likely to result in successful vascular repair, while bigger injuries impeded regeneration. This discovery emphasizes the importance of wound size as a key factor in regeneration efficacy. Understanding how plants respond to various injury sizes might help design strategies to optimize regenerative responses, where minimizing damage and fostering quick recovery are critical for regeneration output.

Another important issue included in the study is the effect of leaf age on regeneration efficiency. the author found out that leaves aged 4-6 days post-germination had the best regeneration efficiency, but older leaves had lower regenerative potential. This age-dependent drop in regeneration demonstrates the dynamic nature of plant regenerative mechanisms and indicates that developmental cues are important in influencing regeneration responses. Further research into the molecular mechanisms underpinning age-dependent alterations in regeneration could provide important insights into the regulatory networks that govern plant development and stress responses.

The positional dependence of regeneration efficiency along the proximodistal axis of the leaf blade is also a fascinating aspect explored in the study. The researchers observed that incisions made at specific positions, such as the junction of the first lateral vein, resulted in

higher regeneration frequencies compared to incisions made at other locations. This positional sensitivity of regeneration outcomes suggests that the spatial organization of vascular tissues within the leaf plays a crucial role in determining regeneration potential. Understanding the spatial dynamics of vascular regeneration could have implications for manipulating leaf architecture and vascular patterning in crop plants to optimize resource allocation and stress tolerance.

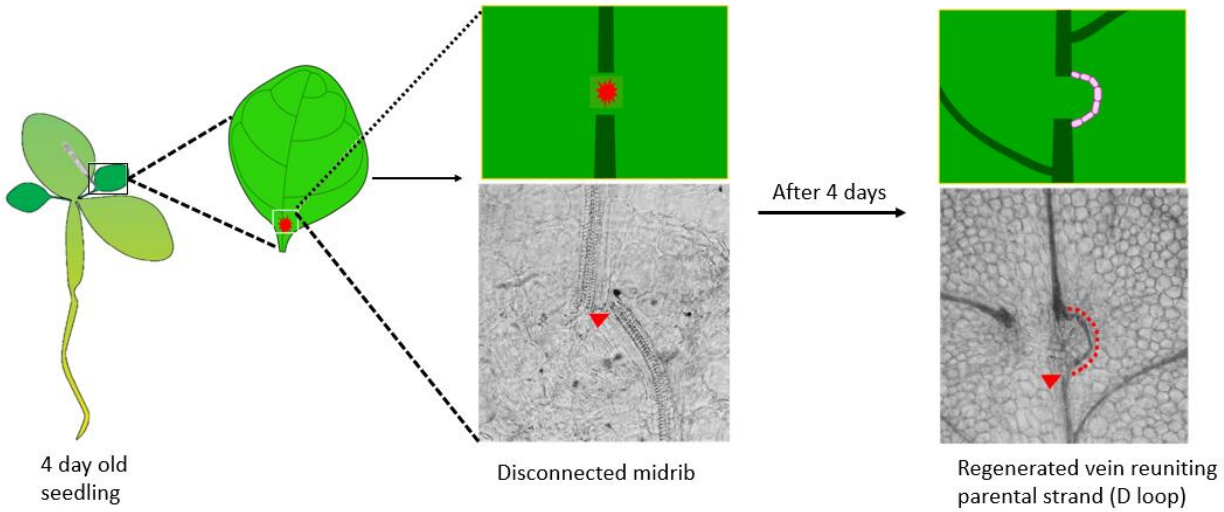

Figure 5: Schematic representing leaf vein regeneration assay. Red star indicates the site of injury in 4 days old seedling of Col-0 wild type. After 4 days of injury, the injury is repaired in the form of a D-loop formed between the disconnected strands (Radhakrishnan et al., 2021).

2 Motivation for the project

After discussing the *de novo* root regeneration there are numerous questions remained unanswered such as what factor govern the touch dependent characteristics of DNRR? What are the factors promoting the leaf explant to generate a new root? Previous investigations demonstrated that the mechanical injury-induced regeneration in *A. thaliana* excised leaf generates adventitious roots when in contact with a substrate or a callus when the cut end is kept untouched. It is also demonstrated that a transcription factor PLT (PLETHORA) such as PLT3, PLT5, PLT7 plays a critical role (Kareem et al., 2015; Radhakrishnan et al., 2020; Shanmukhan et al., 2021) in controlling the formation and maintenance of the root stem cell niche, regulates the expression of genes involved in cell division and signaling molecules that affect root growth and regeneration. Auxin is a plant hormone that regulates cell division, differentiation and is essential for the activation and maintenance of root stem cells and integrates various developmental and environmental cues during plant

regeneration. It has been demonstrated that local auxin response is essential to coordinate DNRR. It is also demonstrated that there is a high auxin level at the cut site in case of touch with the substrate compared to without touch (Figure 6). They also found that *PLT7* expression is high in comparison of with contact compare to without contact (Figure 7). It is interesting that PLT7 overexpression also overrides the need for physical touch during leaf to root regeneration (Shanmukhan et al., 2021) (Figure 8).

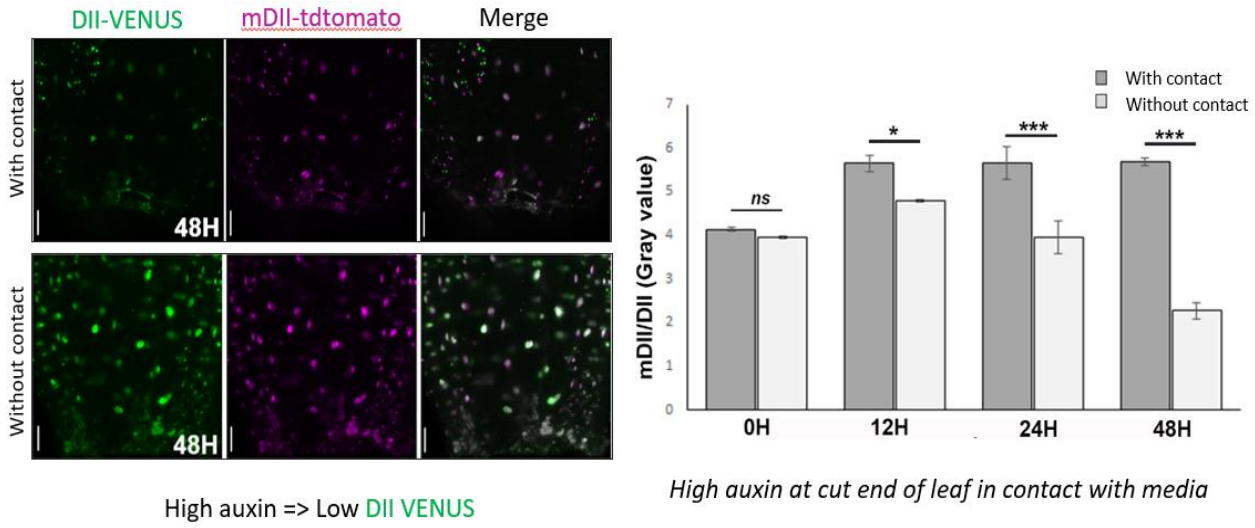

Figure 6: - Time lapse imaging of detached leaves using auxin sensor R2D2 showing auxin response is increased with contact in comparison of without contact with the MS- agar medium surface. (Adapted from Shanmukhan et al., 2021)

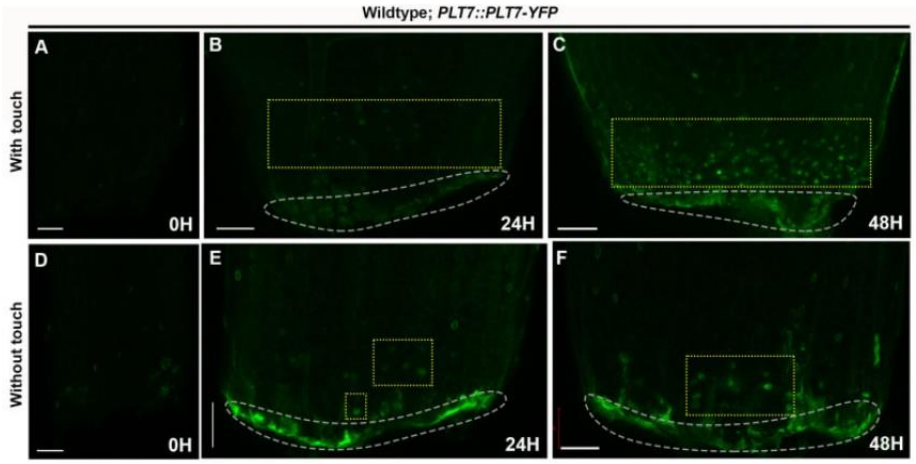

Figure 7: Expression of the *PLT7* gene in With Touch (WT) and in Without Touch (WOT). The numberof cells expressing the *PLT7* signal is more in the WT (A, B, C) as compared to the WOT (D, E, F). H: hours post cut, Adapted from (Shanmukhan *et al.*, 2021).

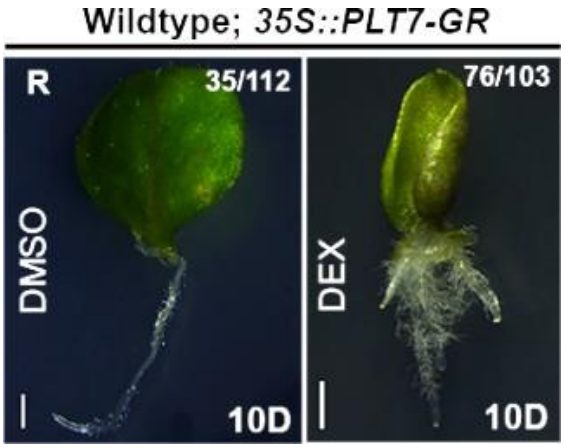

Figure 8: Overexpression of the PLETHORA 7 (PLT7) results in the multiple roots in the Without touch leaf sample. D: days post cut, Adapted from (Shanmukhan et al., 2021)

However, it is not well understood how the PLETHORA 7 and auxin response pathways interact and affect each other during regeneration. Therefore, the main aim of this project is to observe the interplay of the auxin and PLT7 in *A. thaliana* during regenerative response.

2.1 Research design and objectives of the projects

In pursuit of a comprehensive understanding of interplay between PLT7 and auxin, designed two modules within *Arabidopsis thaliana* as the model system. These modules are designed to shed light on the dynamic relationship between PLT7 expression and auxin signaling, particularly in the context of *de novo* regeneration processes.

The first module is dedicated to elucidating the influence of auxin signaling manipulation on PLT7 expression dynamics. To achieve this, a double marker line is constructed, incorporating the *axr3*-1 mutant, well-established as a negative regulator of auxin due to its single amino acid change in the second domain of *AXR3* all this activity is under the control of an inducible promoter (G1090::xve) and the gene is tagged with Red Fluorescent Protein (RFP). Within this line, the endogenous promoter of PLT7 is fused with Yellow Fluorescent Protein (YFP). Through meticulous observation of PLT7 expression patterns in response to auxin signaling perturbations, we endeavor to unravel the nuanced dynamics of their interaction.

In the second module, our focus shifts towards the generation of an RNAi line targeting PLT7, taking advantage of RNA silencing machinery, with the help of the DICER/Argonaute complex, to suppress PLT7 expression. This RNAi line is under the regulation of an inducible promoter (G1090:XVE::PLT7:dsrnai), affording precise

control over the temporal downregulation of PLT7 expression. Subsequent interrogation of the effects of PLT7 knockdown on auxin biosynthesis and signaling pathways is anticipated. The strategic use of an inducible promoter facilitates the delineation of critical temporal windows for auxin-PLT7 interactions during regeneration processes.

Our primary objective is to understand the complicated interplay between Auxin and PLETHORA 7 during *de novo* root regeneration. In addition, we want to look into the dynamics of cell-to-cell communication, the expression kinetics, and the regulatory mechanisms that control auxin modulation and PLT7 expression. We want to understand the interaction of these essential molecular components in plant development through this module design.

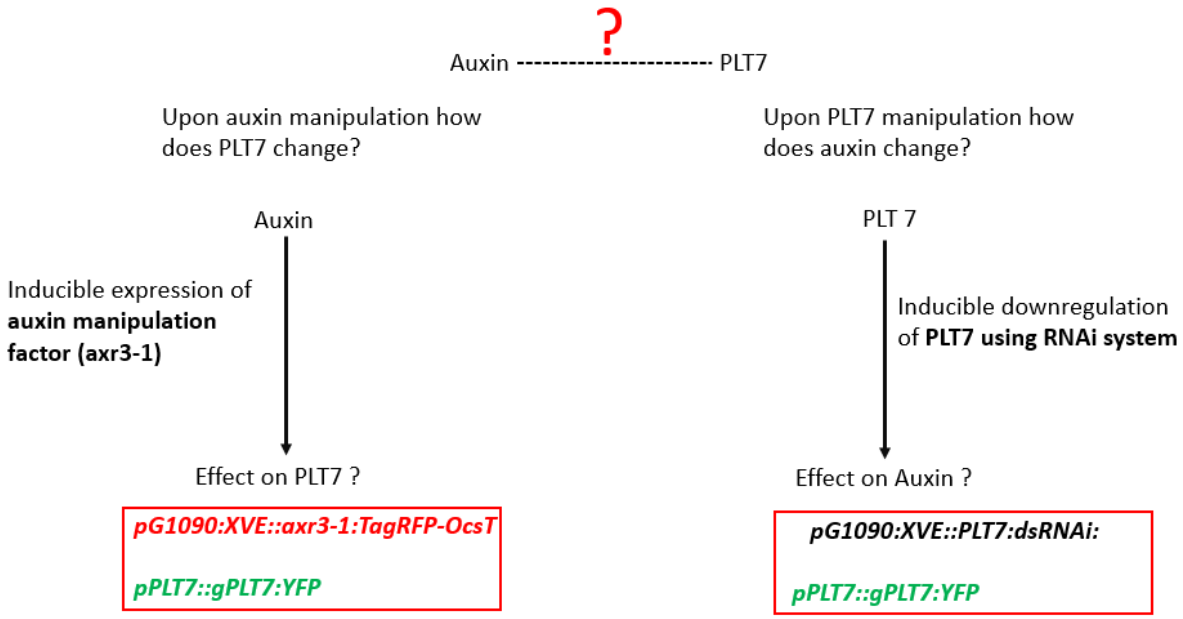

Figure 9: Graphical representation of Modules for Investigating the Interplay between PLT7 and Auxin in *Arabidopsis thaliana*.

2.2 Objective of the project

The main objective of the project is to investigating the interplay of Auxin and PLETHORA 7 during *de novo* regeneration. Moreover, we will check the cell-to-cell interaction, expression pattern, gene expression level in response to auxin modulation and PLT7 knockdown and how this effect each other with the help of the designed modules.

Objective 1: - To check the regeneration response of inducible inhibition of auxin signaling and cytokinin.

1.1: - To investigate the regeneration response of inhibition of auxin signaling and cytokinin at various timepoint during *de novo* root regeneration.

1.2: - To Examine the regeneration response of inhibition of auxin signaling and cytokinin at different timepoint during leaf vein regeneration.

Objective 2: - To explore the relationship between auxin and PLT7 during *de novo* root regeneration.

2.1 construct inducible PLT7RNAi and *axr3*-1 to understand the interaction between auxin and PLT7 during *de novo* root regeneration.

2.2 Generate transgenic lines that can help to investigate the relationship between auxin and PLT7 during DNRR.

Objective 3: - To investigate how PLTs gene expression level affect during inhibition of auxin signaling in DNRR.

3.1 using RT-qPCR to compare the gene expression level of PLT during manipulation of auxin signaling in DNRR.

3.2 using RT-qPCR compare the gene expression level of Autophagy Gene during manipulation of auxin signaling in DNRR.

Objective 4: - To check the expression pattern of PLT7 during inhibition of auxin signaling in DNRR.

Chapter 2 Materials and Methods

2.1 Multisite gateway cloning

The Gateway cloning technology is a popular molecular cloning system that allows for efficient and precise cloning of DNA fragments into plasmid vectors using recombination reactions. A newly developed recombination method called multisite gateway cloning was inspired by the way bacteriophages integrate into bacterial genomes. It accelerates the cloning process by enabling the simultaneous insertion of several DNA segments into a single construct. Using a multisite LR Clonase reaction, the process entails pairing gateway entry clones with binary destination vectors that comprise an promoter (first box), the gene of interest (second box), and terminator/reporter elements (third box).

The protocol of multisite gateway cloning:

1. Design of the DNA sequence:

We designed the DNA sequence of the gene of interest using the snap gene tool. The designed sequence was optimized for expression in our desired host organism.

2. Amplification of DNA fragments:

The DNA fragments corresponding to the gene of interest were amplified using PCR with specific primers containing attB sites. The PCR products were purified using a PCR purification kit and quantified using a spectrophotometer.

3. BP reaction:

The PCR products were cloned into a donor vector using the BP reaction. The BP reaction allows for the creation of an entry clone, which contains the attP sites required for the Gateway cloning system.

4. LR reaction:

The entry clone was then used in an LR reaction with the destination vector, which contains the promoter and other regulatory elements required for gene expression in plants. The LR reaction allows for the transfer of the gene of interest from the entry clone to the destination vector and then finally it will give to the expression clone.

5. Verification of the molecular construct:

The presence and orientation of the DNA fragments in the molecular construct were verified using PCR and restriction digestion.

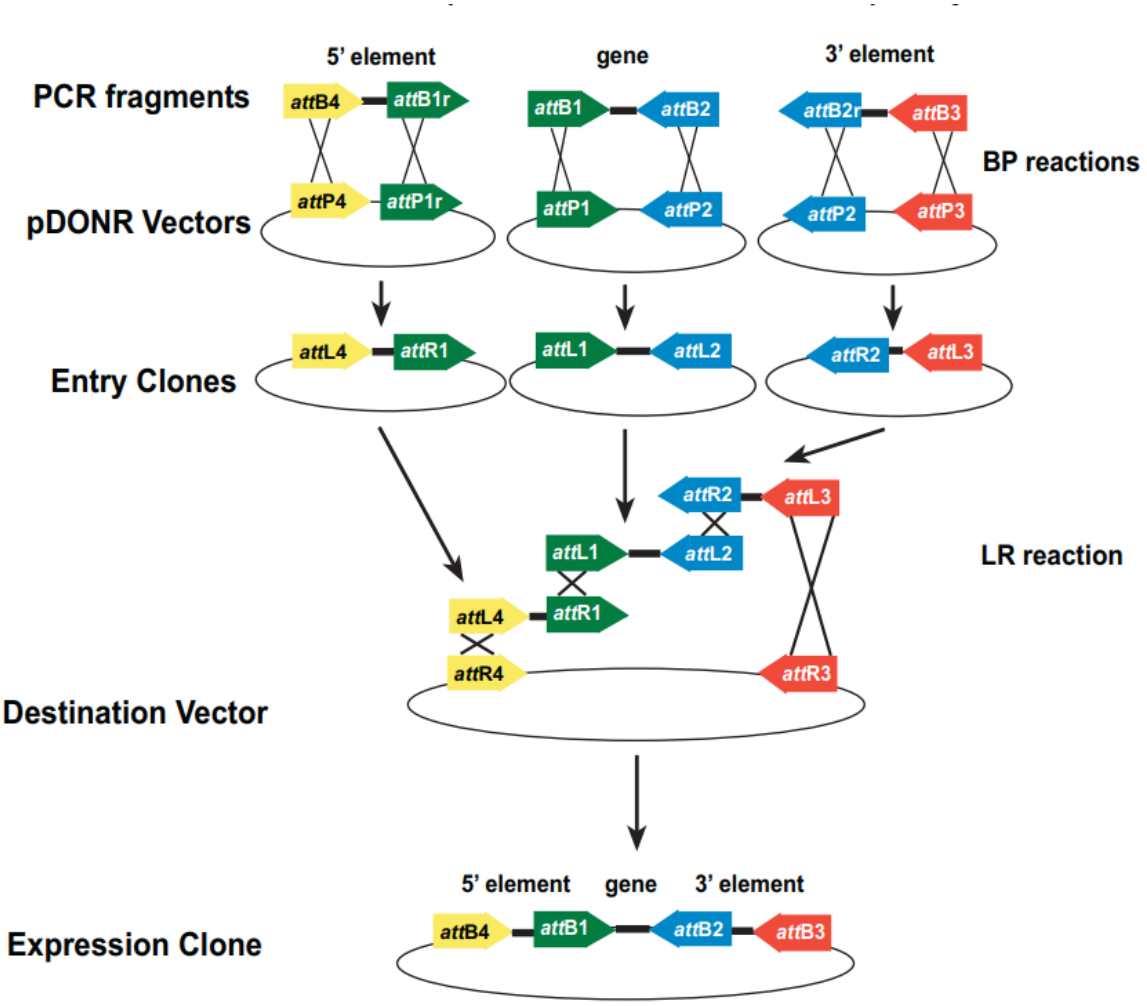

Figure 10: Multisite gateway cloning BP and LR reaction (Invitrogen)

2.2 Seed sterilization and plating:

Surface sterilization of Columbia ecotype *Arabidopsis thaliana* seeds was done using 70% ethanol and 20% bleach. The seeds were then washed with autoclaved distilled water seven times. The sterilized seeds were then given a cold treatment of 2 days to break their dormancy. The seeds were done plated on half-strength Murashige-Skoog (MS) medium (pH 5.7) and the plates were kept vertically at 22°C, under 45µmol/m2 /s in 16hr light and 8hr dark and 70% relative humidity. Around 20 to 30 seeds are plated on one square petri plate (120mm*120mm, Himedia model: PW050-1) containing approximately around 50mL half-MS media.

2.3 Regeneration assays:

(a) *De novo* root regeneration (DNRR): - The growth medium used for the experiment is a Murashige & Skoog medium (MS). To study the *de novo* regeneration response in the detached leaves, the plant leaves of the same age were excised using the scissor and kept on the hormone-free MS Medium. When a detached leaf's abaxial side was placed on the media (i.e., leaf petiole touching the MS medium surface). On the other hand, when the detached leaf's adaxial side was placed on the growth media (i.e., leaf petiole in the air), it produced wound healing or callus.

(b) Leaf vein regeneration: - The date of germination of the seeds was marked as the first day. Four-day old seedlings were used to perform leaf incision/injury. For all the assays, one of the leaves of the first pair of rosettes was injured at lamina petiole junction using a sterile fine tip forceps. The injury was made on the abaxial side to disconnect the mid vein. Care should be taken to avoid injuring the adaxial side. Also, injuries creating a gap of more than 400um cannot be repaired (Radhakrishnan et al., 2020). Immediately after the injury, the plants were kept back in growth chamber under the same growth conditions (22°C, under 45 µmol/m2 /s continuous white light and 70% relative humidity). For assays, the plates were opened after 4 days of injury and the injured leaf from every plant was collected and cleared. Clearing involves dehydration of the sample by increasing ethanol concentration (15%, 50%, 75%, 96%) and then keeping the samples overnight in 100% ethanol. Next day, the samples were again rehydrated by decreasing the percentage of ethanol (96%, 75%, 50%, 15%). Finally, samples were immersed overnight in a solution of chloral hydrate for clearing. Next day, images of cleared leaves were taken using Leica DM6 and a percentage regeneration was scored.

2.4 RNA extraction of DNRR samples

RNA Extraction Protocol Using Macherey-Nagel Kit

step 1: Sample Collection and Freezing

- Collect plant samples and promptly freeze them in liquid nitrogen to preserve RNA integrity.

Step 2: Tissue Homogenization

- Grind the frozen tissue into a fine powder using a mortar and pestle or a tissue homogenizer.
- Transfer approximately 100 mg of the powdered tissue into a sterile microcentrifuge tube.

Step 3: Homogenize Sample

- Grind up to 100 mg tissue under liquid nitrogen.
- Add 350 µL Buffer RA1 and 3.5 µL 8-mercaptoethanol (B-ME) to 100 mg tissue.
- Vortex vigorously.

Step 4: Filtrate Lysate

- Reduce viscosity and clear lysate by filtration through NucleoSpin Filter.
- Place NucleoSpin Filter in a Collection Tube (2 mL).
- Centrifuge for 1 min at 11,000 x g.
- Transfer filtrate to a new 1.5 mL microcentrifuge tube.

Step 5: Adjust RNA Binding Conditions

- Discard NucleoSpin Filter and Add 350 µL ethanol (70%) to the homogenized lysate and mix by pipetting up and down (5 times).

Step 6: Bind RNA

- Take one NucleoSpin RNA Plant Column (light blue ring) placed in a Collection Tube.
- Load the lysate and Centrifuge for 38 s at 11,000 x g.
- Place the column in a new Collection Tube (2 mL).
- Maximum loading capacity of NucleoSpin RNA Plant Columns is 750 µL.

Step 7: Desalt Silica Membrane

- Add 350 µL Membrane Desalting Buffer (MDB).
- Centrifuge at 11,000 x g for 1 min to dry the membrane.
- If column outlet contacts flow-through, discard and centrifuge again for 30 s at 11,000 x g.

Step 8: Digest DNA

- Prepare DNase reaction mixture: 10 µL reconstituted rDNase to 90 µL Reaction Buffer for rDNase and Apply 95 µL DNase reaction mixture onto the center of the silica membrane.
- Incubate at room temperature for 15 min.

Step 9: Wash and Dry Silica Membrane

- 1st wash: Add 200 µL Buffer RAW2, centrifuge for 30 s at 11,000 x g.
- 2nd wash: Add 600 µL Buffer RA3, centrifuge for 30 s at 11,000 x g.
- 3rd wash: Add 250 µL Buffer RA3, centrifuge for 2 min at 11,000 x g to dry the membrane completely.
- Place the column into a nuclease-free Collection Tube (1.5 mL, supplied).

Step 10: Elute RNA

- Elute RNA in 60 µL RNase-free H_2O and Centrifuge at 11,000 x g for 1 min.

If higher RNA concentrations are desired, elution can be done with 40 µL. Overall yield will decrease with smaller elution volumes

cDNA synthesis procedure

Measure RNA concentration and quality of RNA through spectrophotometry and Run samples in gel electrophoresis for quality check. If everything is fine then Set up the cDNA synthesis reaction by creating a master mix with RNA sample, reverse transcriptase enzyme, primers, dNTPs, buffer, and other components. Observe the manufacturer's protocol for volumes and concentrations. Incubate the reaction mixture at recommended temperature for designated time to enable RNA conversion to cDNA. Heat inactivates reverse transcriptase enzyme according to kit instructions. Store cDNA at -20°C or -80°C for long-term or proceed to downstream applications like qPCR or RT-PCR for gene expression studies.

2.5 Microscopy and documentation

For imaging of leaf DNRR samples, Leica S8 APO stereo zoom microscope was used. Confocal images of roots were captured using upright Leica Multiphoton microscope. (standard confocal mode). Confocal laser scanning microscopic time time-point images were acquired using upright Leica Multiphoton confocal laser-scanning microscope. The fixed imaging of DNRR leaves was done using a 10x and 20x air objective. Each timepoint of the DNRR leaves was captured with the same settings. The imaging was performed using 20% laser power (514nm-for YFP and 561nm-for RFP as excitation wavelength), 600-800 master gain and frame size. Filter-sterilized 20µg/ml propidium iodide (PI) was used to stain the cell wall in case of screening of positive transformants. The DNRR leaves images were captured using Leica M205FA stereo microscope. The agarose gels were documented on the Syngene G-box gel doc system.

2.6 Floral dip method

The floral dip method is a popular technique in *Arabidopsis thaliana* for introducing foreign DNA into the plant genome, typically transgenes or mutations. This strategy takes advantage of the plant's natural reproductive activity, namely the creation of flowers and subsequent seed generation. First, the Agrobacterium tumefaciens strain C58C1 bearing the necessary binary vector is created. This strain is frequently modified to have the essential virulence (vir) genes for T-DNA transfer. The binary vector itself includes the gene of interest (GOI) bordered by T-DNA borders, as well as selectable marker genes for plant transformation, such as antibiotic resistance genes. *Arabidopsis* plants are then nurtured under controlled settings until they blossom. It is important to note that the floral dip approach is normally used on plants that haven't yet bolted or grown a flower stalk. The Agrobacterium culture is then cultivated to the desired density in a liquid medium supplemented with appropriate antibiotics for bacterial selection and then incubate at 28 to 29 degree celsius. The culture is then pelleted and resuspended in an infiltration solution containing a surfactant, such as Silwet L-77, to help Agrobacterium adhere to the plant surface. The *Arabidopsis* plants are inverted and immersed in an Agrobacterium suspension containing the surfactant. here, the surfactant helps the bacterial suspension stick to the floral tissues. The entire inflorescence, including flowers and buds, is fully immersed in the suspension for a short time, usually for a minute. After dipping, the plants are gently dried to remove any leftover bacterial suspension. The seeds are

subsequently returned to the growth chamber to mature. If the flowers successfully turn into siliques (seed pods), the seeds within them will transmit the injected DNA. Transformed seeds are extracted and placed on a selective medium containing antibiotic that will not help the non-transformed seeds to grow properly while allowing transformed plants to flourish.

Finally, transgenic lines are tested to ensure transgene incorporation and expression using molecular biology techniques including as PCR and expression analysis. Overall, the floral dip method with Agrobacterium tumefaciens strain C58 is a simple and effective way to introduce transgenes into *Arabidopsis* plants, allowing to better understand gene function and regulation in this model organism.

Chapter 3 Results

Section 3.1 To check the response of auxin and PLETHORA7 during *de novo* root *regeneration*

3.1.1 To Explore the Dynamics of Auxin and PLETHORA7 by Generating Construct and Transformation in *Arabidopsis thaliana*.

Construct 1: pCam(kan) pG1090: XVE::*PLT7-dsRNAi*:

The experimental approach employed in this study involves the creation of an inducible knockdown system targeting the *PLT7* gene. This system utilizes an estrogen receptor-based chemical inducible mechanism that has been specifically optimized for use in transgenic plants. Upon exposure to estradiol in the growth medium, the inducible promoter associated with the construct becomes activated, initiating the transcription process. The resulting transcript comprises both sense and antisense segments derived from the original *PLT7* transcript. This transcript undergoes a unique structural modification, folding into a hairpin loop configuration. This hairpin structure is capable of binding to complementary regions within the original *PLT7* transcript. As a consequence, the RNA silencing machinery, including components such as the DICER/Argonaute complex, is recruited to the site of interaction. Through this mechanism, the expression of the PLT7 gene is effectively inhibited. The utilization of an inducible promoter confers several advantages to this system. Firstly, it offers precise control over the timing and magnitude of gene expression, enabling to regulate PLT7 expression as needed for their experiments. Moreover, the cell type-specific properties of this system minimize the risks associated with unintended or ectopic gene expression, thereby broadening its applicability across different experimental contexts. In this study, an XVE-based estrogen-inducible promoter is selected for its favorable characteristics. This promoter exhibits low baseline expression levels and minimal constitutive activation, ensuring tight control over PLT7 expression. Importantly, the inducible system demonstrates negligible disruptions to physiological processes and developmental pathways, making it suitable for use in diverse research environments with minimal impact on plant health and function. Firstly, I did PCR amplify each of your DNA fragments separately, including promoter sequence, the PLT7 sense gene element, and the PLT7 antisense sequence, using primers that add attB sites to the ends of each fragment. These attB sites serve as recombination sites for the Gateway cloning system.

Next, I performed BP recombination reactions to transfer each PCR-amplified fragment into a corresponding Entry vector containing the appropriate attP sites. Each fragment would now reside in its own Entry clone. With the help of bp clonase enzyme and after that we digest the individual entry clones vectors with the restriction enzyme to confirm. Subsequently, I performed a Multisite LR recombination reaction, combining your promoter sequence, PLT7 sense gene element, and PLT7 antisense sequence from their respective Entry clones, along with a destination vector containing attR sites Pcam (kan,kan). This LR reaction results in the assembly of all the fragments into the destination vector, generating your final construct.

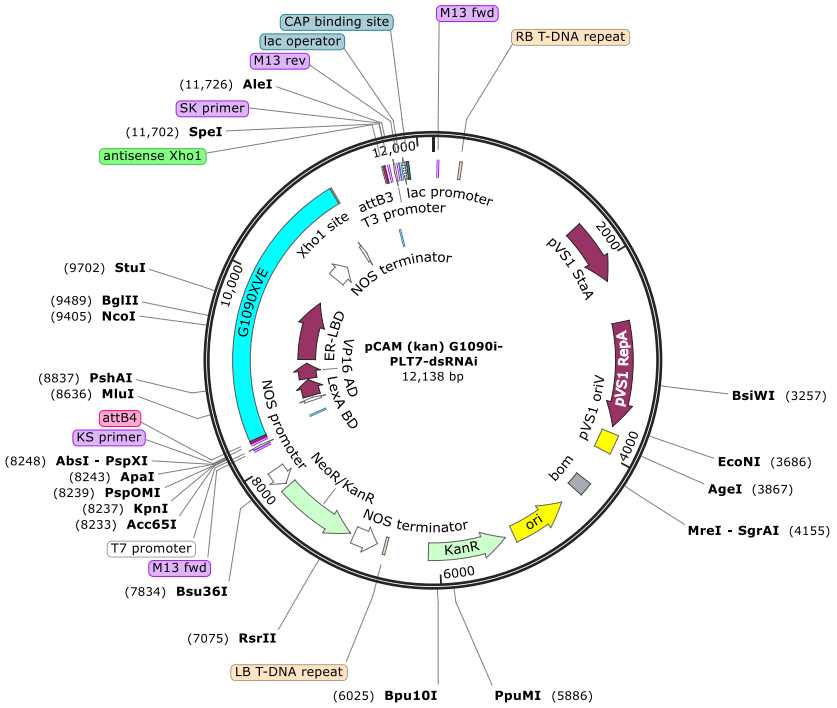

Figure11: Plasmid map of pCam*(kan) pG1090:XVE::PLT7:dsRNAi*

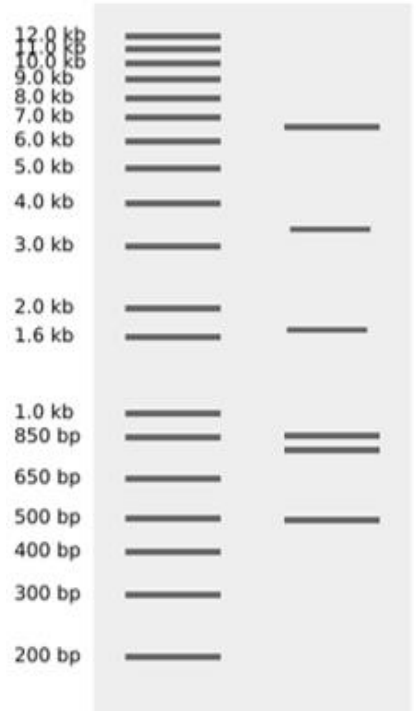

Figure12 : Expected banding pattern of virtual digested *pCam(kan) pG1090:XVE::PLT7 dsRNAi* with EcorI and HindIII

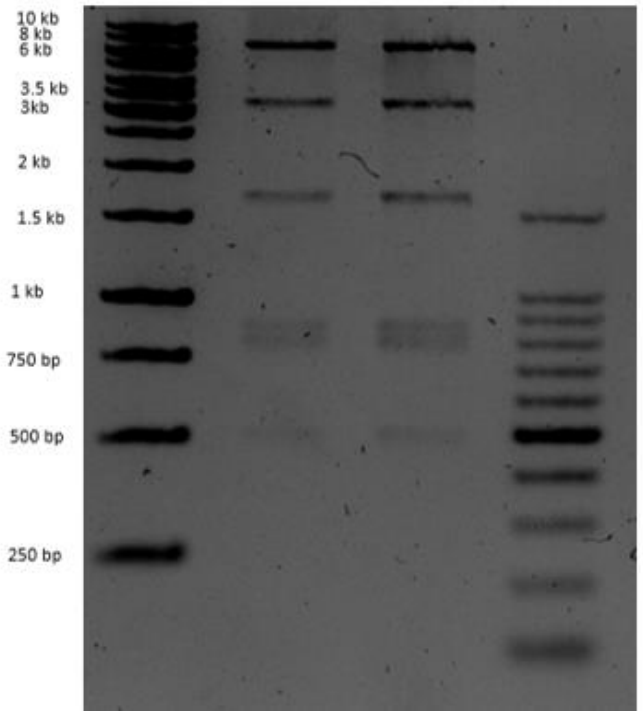

Figure13: Expected banding pattern of digested *pCam(kan) pG1090:XVE::PLT7 dsRNAi* with EcorI and HindIII

Construct 2: - pCam (kan) pG1090: XVE::*axr3*-1:TagRFP-OcsT:-

The *AXR3* gene (AUXIN RESISTANT 3), plays a critical role by regulating auxin response. Auxin, regulates various growth processes like cell elongation and differentiation and many more. *AXR3* functions as a negative modulator of auxin signaling pathways. Its activation accelerates the degradation of transcriptional regulators termed AUX/IAA proteins, consequently impeding auxin-mediated responses. These AUX/IAA proteins typically repress the activity of transcription factors responsible for governing the expression of auxin-responsive genes. Through its action, *AXR3* facilitates the activation of these transcription factors, thereby promoting the expression of auxin-responsive genes. This regulatory mechanism impacts diverse biological activities such as root development, vascular tissue differentiation, and tropic responses like gravitropism and phototropism. Mutations in the *AXR3* gene can change the response towards auxin, resulting in abnormal growth phenotypes in plants. For example, loss-of-function mutations in *AXR3* can cause increased sensitivity to auxin, leading to exaggerated responses such as enhanced root elongation and increased lateral root formation. Conversely, overexpression of *AXR3* can lead to reduced sensitivity to auxin and phenotypes such as decreased root growth and altered leaf morphology. All things considered, the *AXR3* gene is essential for controlling auxin responses, which in turn controls many facets of plant growth and development. the *AXR3* gene is essential for controlling auxin responses, which in turn controls many aspects of plant

growth and development. In this case the first box is inducible promoter (pG1090.xve) and the second box is the main gene body i.e. (*axr3*-1). This estradiol inducible construct will overexpress the *AXR3* gene, known to suppress auxin signaling. The fluorescent TagRFP-OcsT protein allows visualization of *AXR3* expression and localization within cells. *AXR3*, also known as IAA17, belongs to Aux/IAA protein family (Ouellet et al., 2001). In second domain of *AXR3*, there is a single amino acid change gives the result *axr3*-1 acts as a negative regulator of auxin signaling. Firstly, PCR amplification was carried out for each DNA fragment separately, encompassing the G1090.XVE promoter sequence, the AXR3 gene element, and the TAG RFP box element, utilizing primers designed to introduce attB sites at the ends of each fragment. These attB sites facilitate subsequent recombination steps in the Gateway cloning system. Following PCR amplification, BP recombination reactions were executed to transfer each PCR-amplified fragment into an Entry vector harboring the corresponding attP sites. This process yielded individual Entry clones, each containing one of the DNA fragments. Verification of successful recombination was achieved by digesting the individual Entry clones with restriction enzymes. Subsequently, a Multisite LR recombination reaction was conducted, amalgamating the G1090.XVE promoter sequence, AXR3 gene element, and TAG RFP box element from their respective Entry clones. These fragments were then integrated into a destination vector housing attR sites, specifically designed for the final construct. The LR reaction catalyzed the assembly of all fragments into the destination vector, culminating in the generation of the desired construct and then I did the bacterial transformation using DH5α strain and isolate the plasmid and digest with the restriction enzymes to confirm.

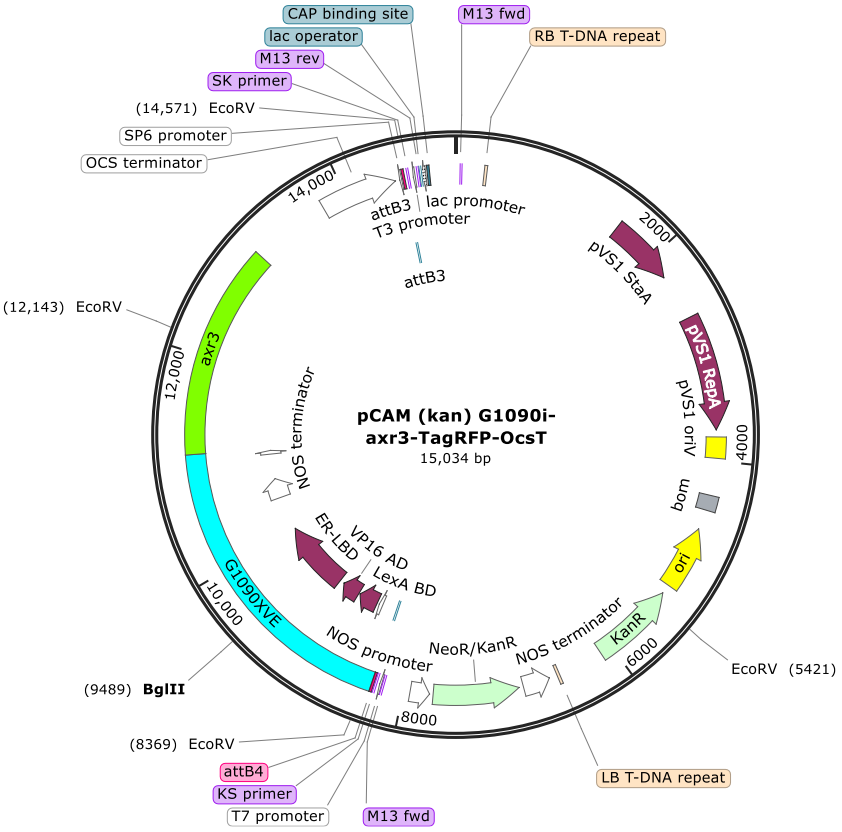

Figure14: Plasmid map of pCam*(kan) pG1090:XVE::axr3-1:TagRFP-OcsT*

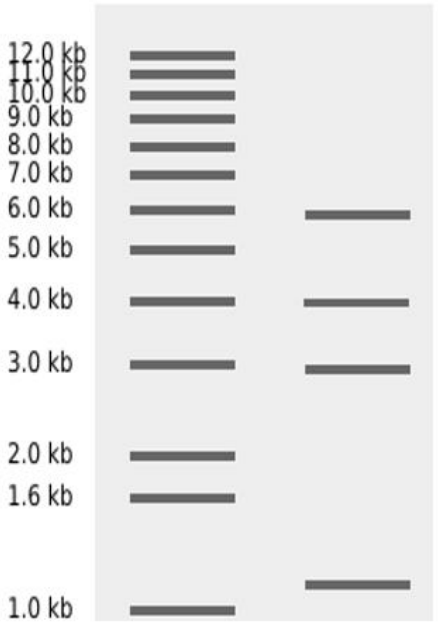

Figure15: Expected banding pattern of virtual digestion *pCam(kan) pG1090:XVE::axr3-1:TagRFP-OcsT digested* with *EcorV and BglII*

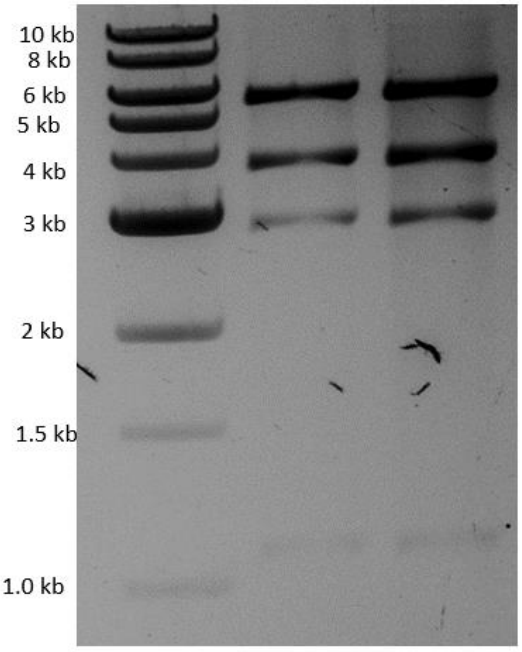

Figure16: Expected banding pattern of *pCam(kan) pG1090:XVE::axr3-1:TagRFP-OcsT digested* with *EcorV and BglII*

Construct3: - pFRm43GW pPLT7::gPLT7: mNG:NosT: - The construct is a translational reporter of *PLT7*. The vector (pFRm43GW) contains the 5kb *PLT7* promoter upstream of the translation start codon and the *PLT7* genomic sequence fused with a green fluorescent protein (GFP) for visualizing expression and localization within cells. This construct can be deployed to observe *PLT7* localization and expression during plant growth and development. Translation fusion reporter constructs are useful for a variety of applications, including investigating the subcellular localization of a protein, studying the temporal and spatial patterns of gene expression, and identifying regulatory elements that control gene expression. The vector backbone contains a seed coat RFP marker to facilitate transgenic selection by fluorescence.

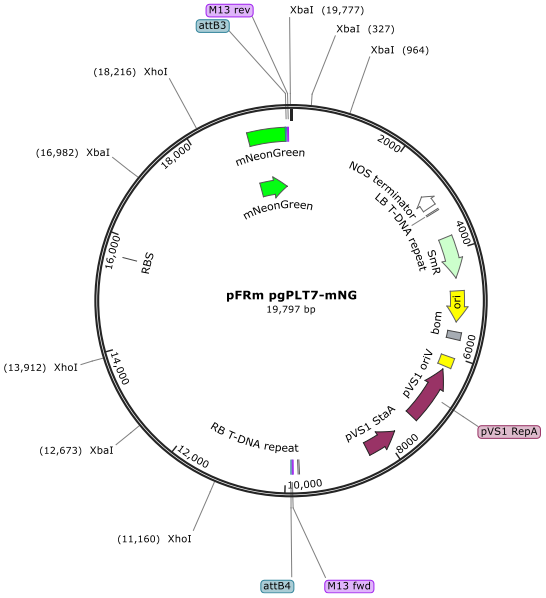

Figure17: Plasmid map of pCam*(kan) pFRm43GW pPLT7:gPLT7: mNG:NosT*

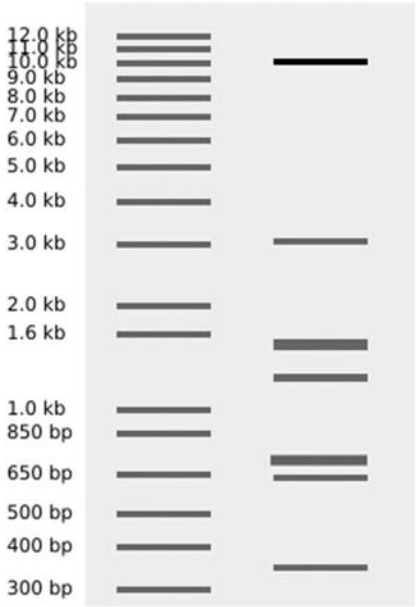

Figure18: Expected banding pattern of virtual digestion *pFRm43GW pPLT7:gPLT7: mNG:NosT digested* with xho1 *and xba1*

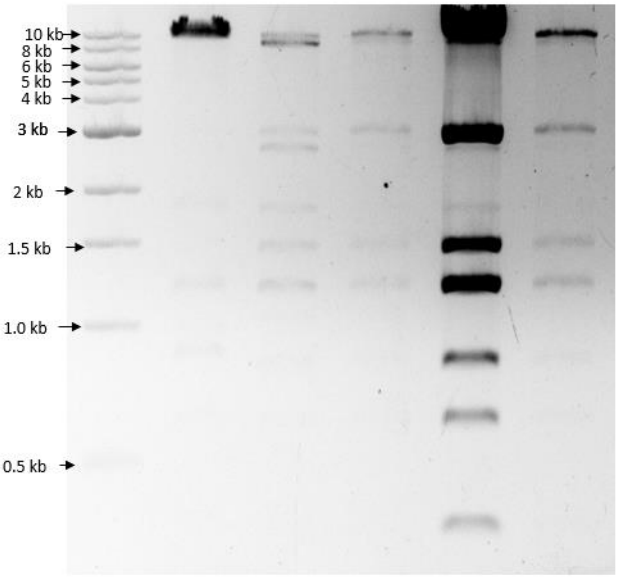

Figure19: Expected banding pattern of virtual digestion *pFRm43GW pPLT7:gPLT7: mNG:NosT digested* with xho1 *and xba1*

3.1.2 screening of positive transformants using seed coat marker

In the model plant *Arabidopsis thaliana*, the *OLE1* gene plays a significant role as a marker for seed coat development due to its encoding of a crucial enzyme involved in oleic acid synthesis. OLE1 shows specific expression in the seed coat tissue, making it a valuable tool for studying seed coat processes. Researchers can visualize OLE1 promoter activity in the seed coat by using reporter genes like GUS or GFP or RFP.

In our case the destination vector has the OLE1 promoter and OLE1 genomic part and then tagged with the fluorescent RFP marker. This whole thing in the destination vector (PFrm43GW) denoted as fastred cassette in the plasmid map. We used this for screening the seeds or select the positive transformants after the floral dip as mentioned in the material and method section.

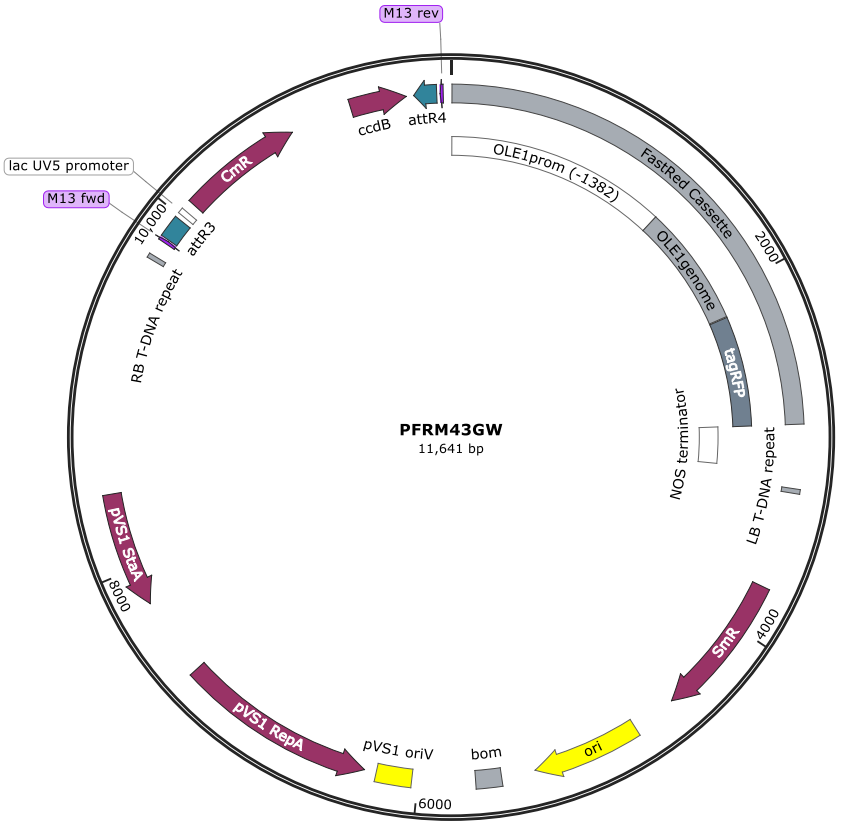

Figure20: Plasmid map of *pFRm43GW* which has a seed coat marker

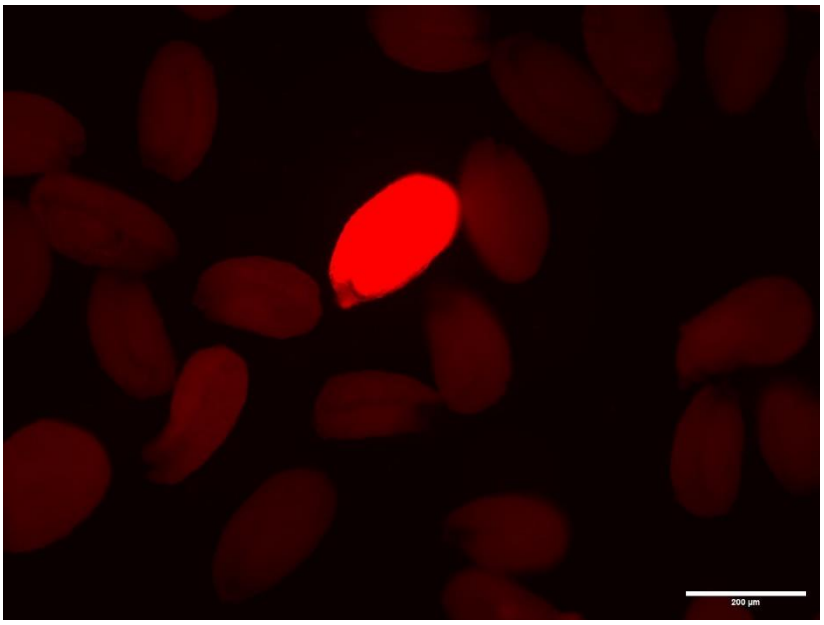

Figure 21: Image of the *Arabidopsis* seeds in the stereo zoom microscope which has a seed coat RFP marker. (scale bar = 200um)

3.2 Hormonal regulation of *de novo* root regeneration and Leaf vein regeneration in *Arabidopsis thaliana*: -

3.2.1 To check the effect of inhibition of auxin signaling and cytokinin in case of with contact to the media during *de novo* root *regeneration*.

Phytohormones like auxin and cytokinin are essential for normal plant growth and development, determining organ formation identity and extent in *de novo* organogenesis based on their ratio. We study how dynamic fluctuations in the level of endogenous hormones such as auxin and cytokinin affect the regeneration process of *de novo* root regeneration. For the study, we have used transgenic lines for the transient overexpression of the genes, which involves the imbalance of these hormones.

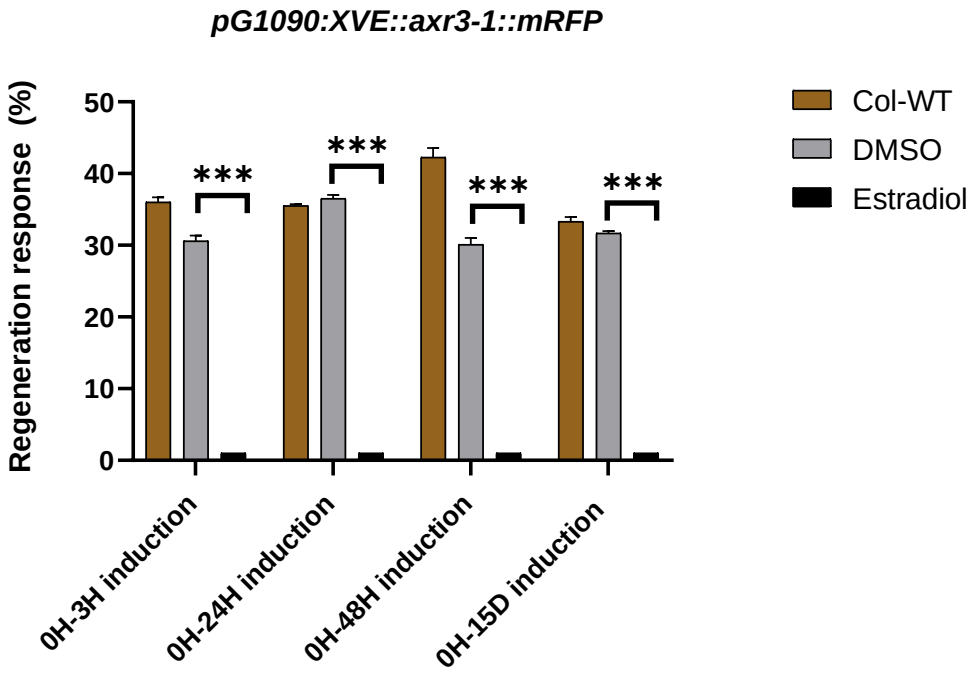

Figure 22: Graph depicting the regeneration efficiency of *de novo* root regeneration by continuous induction of *axr3*-1 at different timepoint. Here the technical replicate is 3. Error bar represent standard error mean (s.e.m). (****P<0.0001, two ANOVA comparison test)

Our studies indicate that any disturbance in the subtle hormonal balance hampers *de novo* root regeneration efficiency drastically. For the study, we used inducible lines, as these hormones are very crucial for the plants. The advantage of inducible lines that we can manipulating these hormones at any given point of times so that we can easily figure it out at which time frame these hormones are playing crucial role. The promoter is an estrogen receptor-based chemical inducible system that is optimized for transgenic plants, so upon estradiol induction in media, the promoter is activated, and for control I used dimethyl sulfoxide (DMSO) as solvent control. For manipulating of auxin signaling, we use (pG1090: XVE::*axr3*-1-RFP). An inhibition of auxin signaling results in drastic reduction in regeneration. Similarly, for cytokinin degradation we used pG1090: XVE::CKX3-YFP transgenic line and found out that a decrease in the cytokinin concentration results in drastic reduction in regeneration of *de novo* root regeneration. In case of auxin signaling manipulation, I did the experiment with the continuous induction at different timepoint which involve continuous induction till scoring (i.e. 15 days post cut), 24Hr continuous induction, 3Hr continuous induction, 48Hr continuous induction and transient induction of 15 minute after different timepoint at 15 minute induction immediately after cut, after 24Hr post cut induction, 3Hr post cut induction to figure it out that at which time point auxin signaling is playing crucial

role for *de novo* root regeneration.

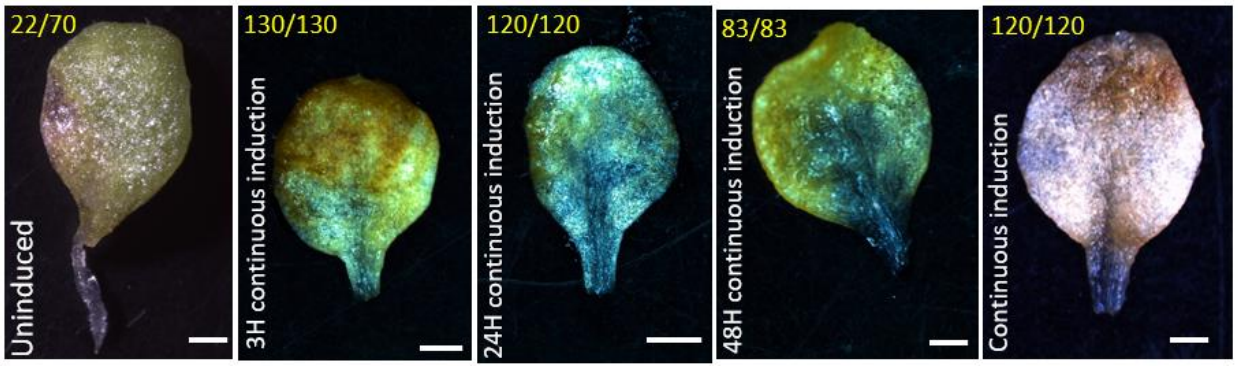

Figure 23: Representative stereo imaging of axr3-1 uninduced and continuously induced leaf at different timepoint (scale bar = 2mm).

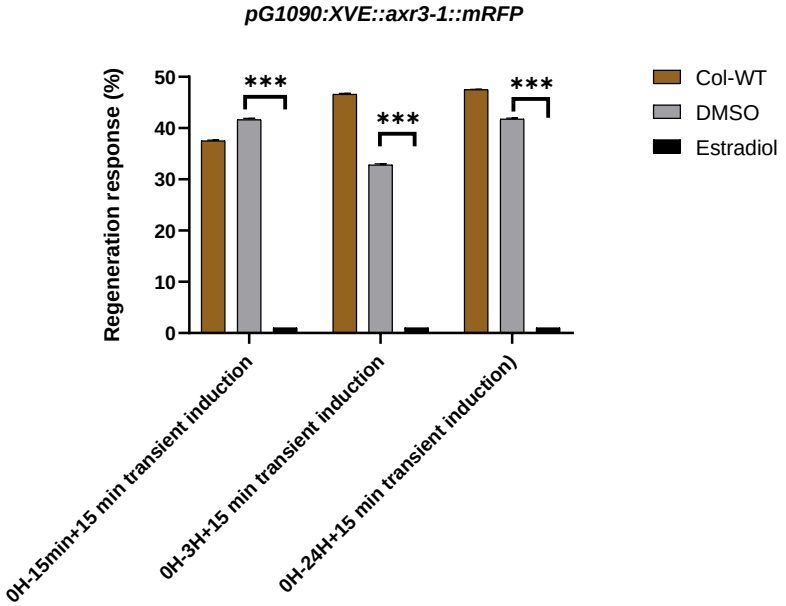

Figure 24: Graph depicting the regeneration efficiency of *de novo* root regeneration following by 15-minute transient induction of *axr3*-1 at different time points. (HPC = hours post cut). Here the technical replicate is 3. Error bar represent standard error mean (s.e.m). (****P<0.0001, two ANOVA comparison test)

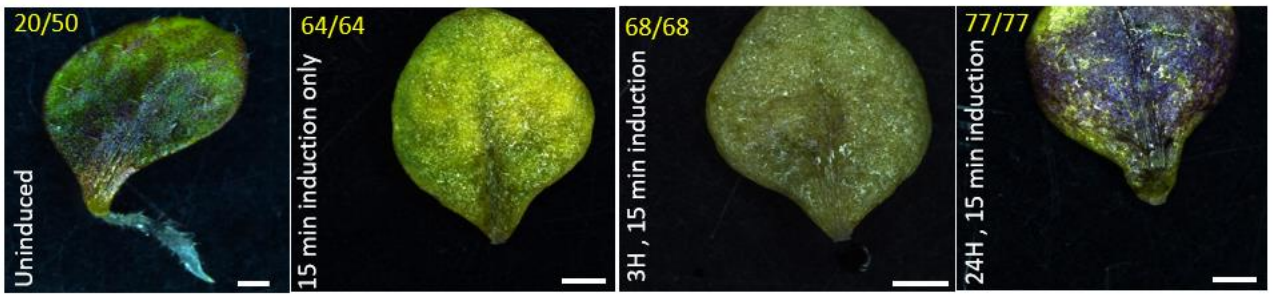

Figure 25: Representative stereo imaging of axr3-1 uninduced and 15 minutes of transiently

induced leaf at different timepoint. (HPC = hours post cut) (scale bar = 2mm).

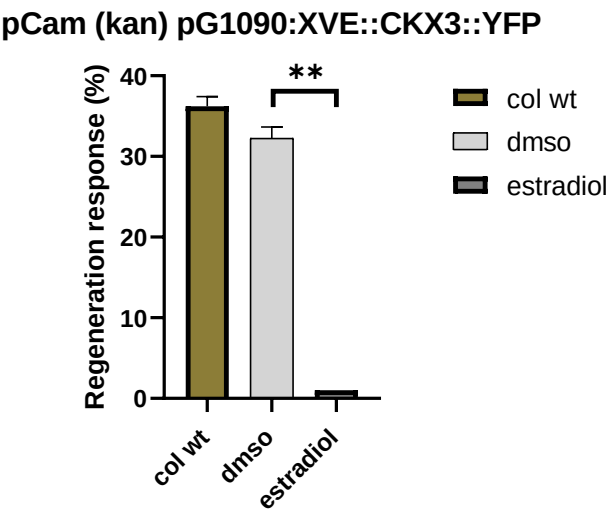

Figure 26: Graph depicting the regeneration efficiency of *de novo* root regeneration by inducing CKX3 with continuous induction. (Pearson's χ^2 test used to find out the significance between dmso and estradiol treatments.)

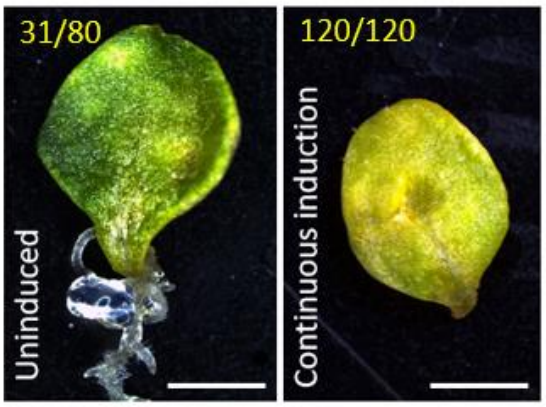

Figure 27: Representative stereo imaging of ckx3 uninduced and continuous induced leaf (scale bar = 2mm).

From our study, we observed that the overexpression of *axr3*-1 which hamper auxin signaling completely abolished the *de novo* root regeneration. we observed this kind of phenomena at different timepoint. Interestingly not only in the continuously induced samples but also transiently induced samples were showing completely abolished regeneration. Even a 15-minute transient induced samples were showing completely no sign of regeneration that means any hamper or hinder in auxin signaling leads to completely abolished the regeneration in *de novo* root regeneration. Similarly, In case of overexpression of CKX3 which degrades cytokinin completely abolished the *de novo* root regeneration from that we can conclude that cytokinin is also playing a very

crucial role in *de novo* root regeneration.

In conclusion, the data shows that any disturbance in auxin or cytokinin levels leads to a severe effect on regeneration. During *de novo* root regeneration, the interplay between the phytohormones auxin or cytokinin plays a crucial role for the formation of *de novo* root regeneration.

3.2.2 To check the effect of inhibition of auxin signaling in case of without contact to the media during *de novo* root *regeneration*.

To investigate the regeneration response in *Arabidopsis thaliana* using pG1090:XVE::*axr3*-1-RFP in case of without touch or contact. The samples were induced only for 15 minutes at different timepoints which involve at 15-minute induction immediately after cut, after 24Hr post cut induction, 3Hr post cut induction. The idea behind choosing time point like this was to figure it out that at which time point auxin signaling is playing crucial role for regeneration in case of wound healing or callus formation. From the observation of our study there is no such big difference in case of wound healing or callus formation between after 3 hour post cut induction and 24 hours post cut induction but there is a less wound healing response in case of initial 15 minutes induction immediately after cut that means initial disturbing of auxin signaling can hamper or reduce the wound healing response. Interestingly, the size of the callus size is bigger in case of uninduced (dmso) compare to the induced (estradiol) across all the timepoints.

In summary we can conclude from this study that perturbation in auxin signaling is affecting severely in case of *de novo* root regeneration response but not wound healing response.

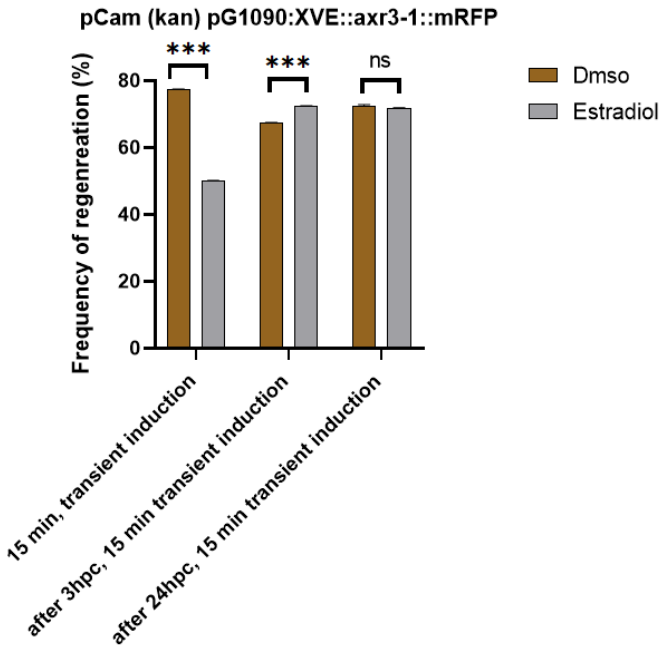

Figure 28: Graph depicting the regeneration efficiency in *Arabidopsis thaliana* in case of non-touch or without contact by inducing *axr3*-1 at different timepoint with 15 minutes of transient induction. Here the technical replicate is 3. Error bar represent standard error mean (s.e.m). (****P<0.0001, two ANOVA comparison test)

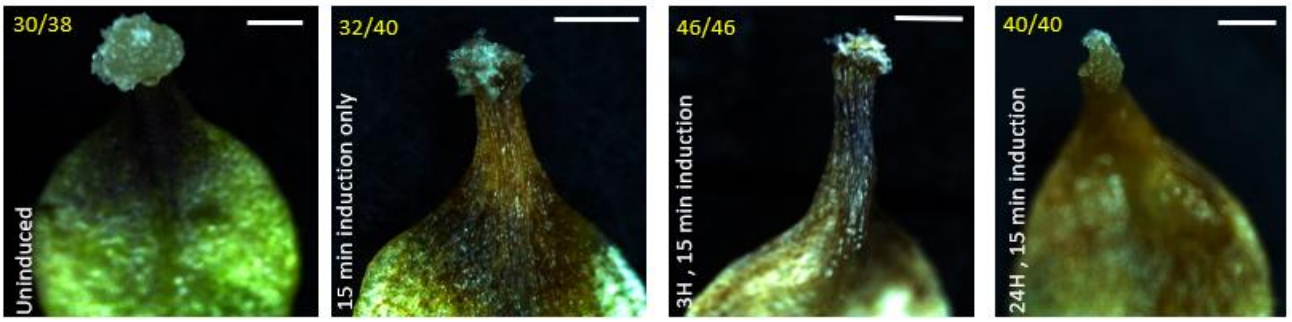

Figure 29: Representative stereo imaging of axr3-1 uninduced and 15 minutes of transiently induced leaf in non-touch or without contact with media at different timepoint. (HPC = hours post cut) (scale bar =2mm).

3.2.3 To check the effect of inhibition of auxin signaling and cytokinin during leaf vein regeneration.

Hormone signaling is an important aspect in shaping both developmental processes and regeneration. Within the realm of plant biology, there are two fundamental hormones, auxin and cytokinin, that assume central roles in the process of leaf vein regeneration. Auxin acts as a crucial regulator of tissue differentiation and patterning. In this process, auxin is an important signaling molecule that influences cell division, elongation, and differentiation in leaf tissues. It starts regeneration by activating meristematic cells near the damaged area, which can develop into various cell types necessary for vein regrowth. Additionally, auxin promotes the growth of meristematic

cells near the injured site, creating a pool of undifferentiated cells crucial for forming new vascular tissue, auxin gradients serve as spatial cues that provide precise organization of nascent vascular tissue, ensuring its alignment with pre-existing vascular networks within the leaf. Auxin is crucial for directing proliferating cells to become specialized cell types like procambial cells, following established auxin gradients. Auxin creates a favorable environment for cell growth and differentiation in regeneration. Cytokinin assists auxin in leaf vein regrowth by maintaining and activating meristems, promoting cell division, and boosting vascularization. It aids in meristematic cell proliferation near the injury site, ensuring a constant supply of undifferentiated cells for new vascular tissue formation. Additionally, cytokinin guides procambial cell transformation into mature vascular components like xylem and phloem, essential for effective vein regeneration. By regulating gene expression and cellular development, cytokinin helps establish a functional vascular system in the regenerating leaf, vital for nutrient and water distribution to support overall leaf health.

For our study we used pG1090:XVE::*axr3*-1-RFP and pG1090:XVE::*CKX3*-YFP for perturbing in auxin signaling and cytokinin degradation and through this experiment we want to know how leaf vein regeneration responds to degradation of cytokinin and perturbation of auxin signaling and how this will affect the overall efficiency of the regeneration. From our study we can concluded that the leaf vein regeneration is hypersensitive to auxin signaling in first 24 hours and same goes with the cytokinin initial 24 hours is very sensitive. Any disturbance in auxin signaling and cytokinin in the first 24 hours can lead to drastically decrease in the leaf vein regeneration.

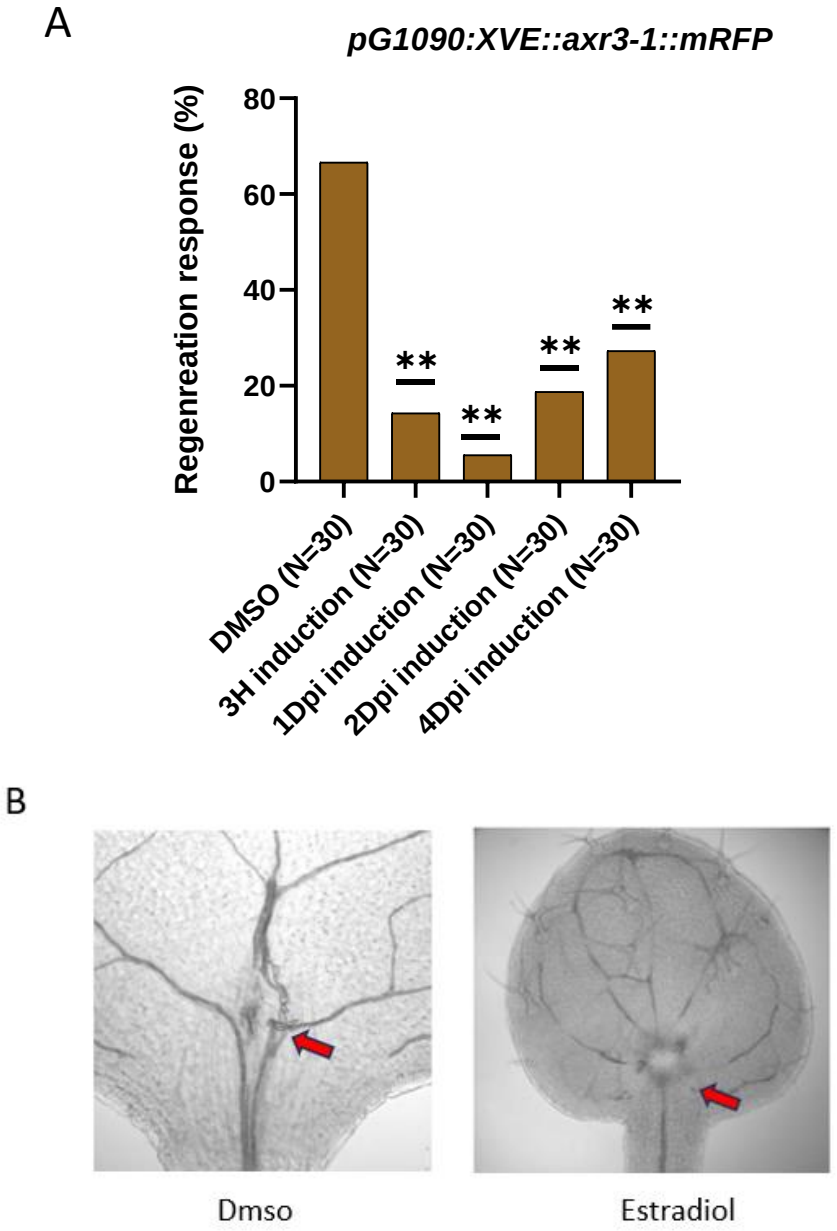

Figure 30: (A) Graph depicting the regeneration efficiency of leaf vein regeneration by inducing *axr3*-1 at different timepoint with continuous induction. (B) Representative images of *axr3*-1 in dmso (control) where leaf vascular regenerate in form of D-loop and estradiol (treatment) where leaf vascular doesn't show any repair or regeneration. (Pearson's χ^2 test used to find out the significance between dmso and estradiol treatments.)

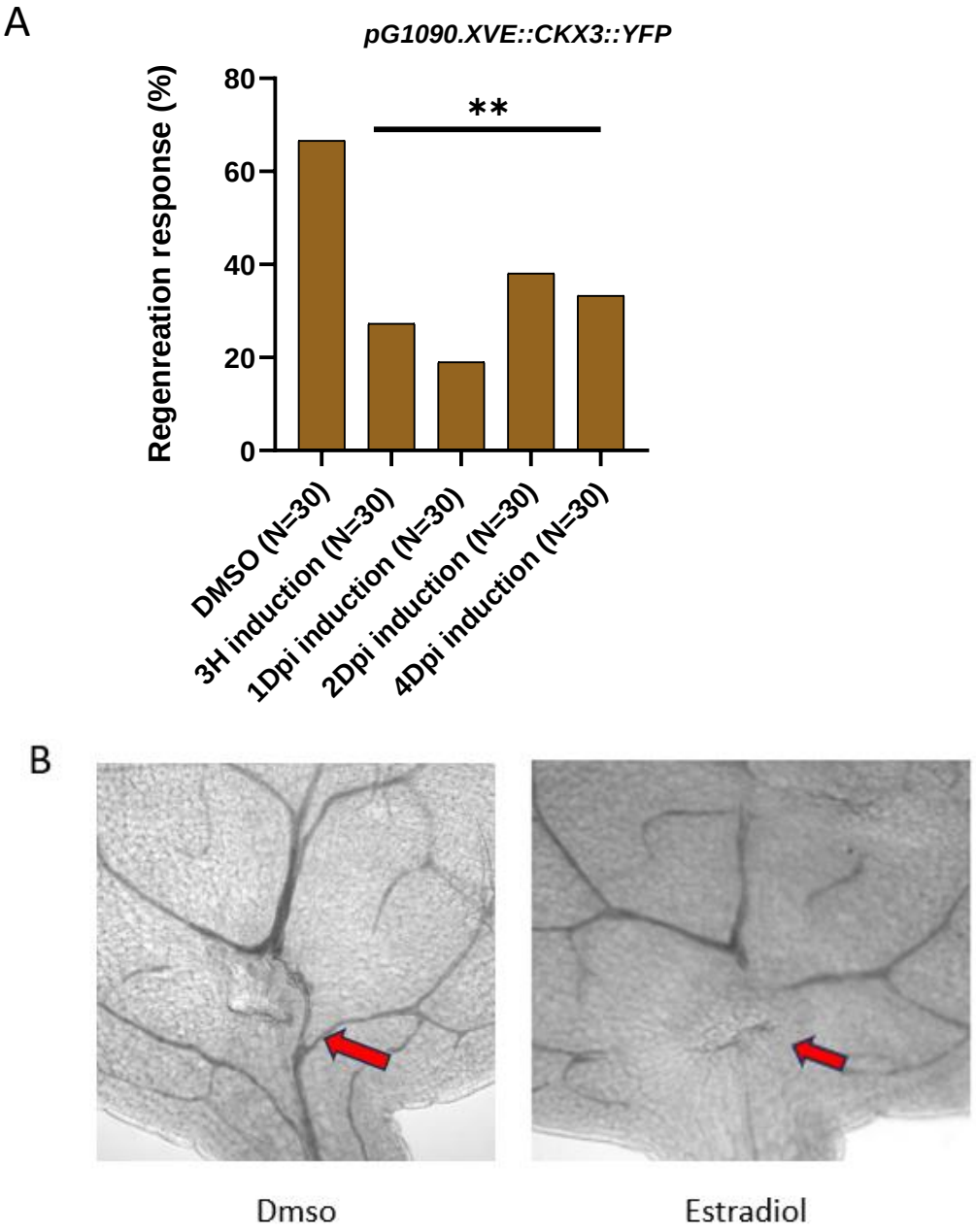

Figure 31: (A) Graph depicting the regeneration efficiency of leaf vein regeneration by inducing Ckx3 at different timepoint with continuous induction. (B) Representative images of Ckx3 in dmso (control) where leaf vascular regenerate in form of D-loop and estradiol (treatment) where leaf vascular doesn't show any repair or regeneration. (Pearson's χ^2 test used to find out the significance between dmso and estradiol treatments.)

3.2.4 Screening of double marker transgenic lines.

Analysis of Dual Marker Transgenic Lines Expressing pPLT7::gPLT7:YFP and pG1090i::*axr3*-1:mRFP.

For the purpose of this project, I generated double marker lines, which will be useful in understanding the interplay between PLT7 and auxin. A transgenic *Arabidopsis* lines expressing the *PLT7*-YFP and *axr3*-1-RFP were generated and screened for their expression patterns within *Arabidopsis* root tissue. Both of them are expressed in the nucleus. *PLT7* is expressed only in the lateral root primordia where few cells express *PLT7*, whereas *axr3*-1 is also expressing in the nucleus of the cells but the expressing localization is all over the root tissue. From the help of this double marker line, it can figure out that how any perturbation in auxin signaling affecting the *PLT7* and how this will overall affect the *de novo* root regeneration. The promoter of the *axr3*-1 is an inducible promoter. For screening of this lines, I used ½ MS media with estradiol in it and then induced it for 12 hours and for control I used DMSO as a solvent control to ensure that there is not a leaky expression of the *axr3*-1 in the tissue.

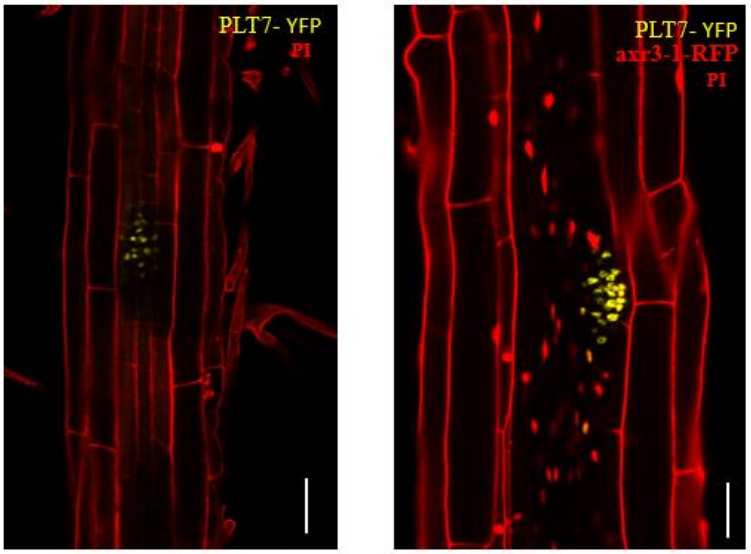

Figure32: Representative confocal images of *Arabidopsis thaliana* root expressing pPLT7::gPLT7:YFP (yellow) in case of DMSO (control) and in case of estradiol (treatment) samples were induced for 12 hours *Arabidopsis thaliana* root expressing both pPLT7::gPLT7:YFP (yellow in colour) with pG1090:XVE:: *axr3*-1:mRFP (red in colour).

Analysis of Dual Marker Transgenic Lines Expressing DR5rev::3XVenus-N7 and pG1090i::*axr3*-1: mRFP.

I generated a parent DR5-Venus auxin reporter line in conjunction with the *axr3*-1 mutant to investigate how auxin signaling impacts tissue-wide auxin distribution. This

line allows for exploration of the spatial and temporal changes in auxin signaling in the *axr3*-1 setting. This will help to trace the overall localization pattern in case of *de novo* root regeneration. More importantly this will also help also help in leaf vein regeneration where the gradient of auxin plays a crucial role it is very interesting to see how auxin signaling perturbation is impacting overall auxin distribution in leaf vein regeneration. For screening of this lines, I used ½ MS media with estradiol in it and then induced it for 12 hours and for control I used DMSO as a solvent control to ensure that there is not a leaky expression of the *axr3*-1 in the tissue.

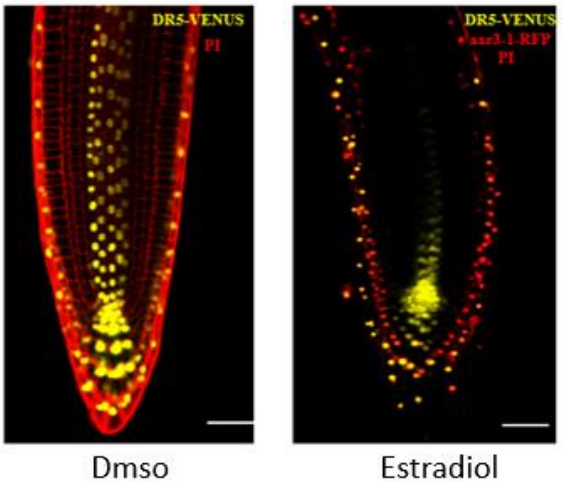

Figure33: Representative confocal images of *Arabidopsis thaliana* root expressing DR5rev::3XVenus-N7 (yellow) in case of DMSO (control) and in case of estradiol (treatment) samples were induced for 12 hours *Arabidopsis thaliana* root expressing both DR5rev::3XVenus-N7 (yellow in colour) with pG1090:XVE:: *axr3*-1:mRFP (red in colour).

Analysis of Dual Marker Transgenic Lines Expressing pPLT3::gPLT3:YFP and pG1090i::*axr3*-1:mRFP.

Plants adopt various modes of natural regeneration to heal wounds occurring during their sessile lifespan. In this project, we explore how PLT7 responds to perturbing auxin signaling. Although it is well-known that three redundant transcription factors, PLETHORA (PLT) PLT3, PLT5, and PLT7, are essential for wound healing responses and *de novo* organogenesis in growing plants and detached organs, respectively. The cumulative loss of function of these genes leads to compromised wound healing responses and *de novo* regeneration in *Arabidopsis*. For *de novo* root regeneration, PLT3 and PLT7 play a crucial role. From previous data in our lab, PLT7 is transcriptionally activated by PLT3, so I generated a line PLT3-YFP with the conjugation of *axr3*-1. From this line, we can observe how PLT3 responds to the manipulation of auxin signaling. For screening

of this lines, I used ½ MS media with estradiol in it and then induced it for 12 hours and for control I used DMSO as a solvent control to ensure that there is not a leaky expression of the *axr3*-1 in the tissue.

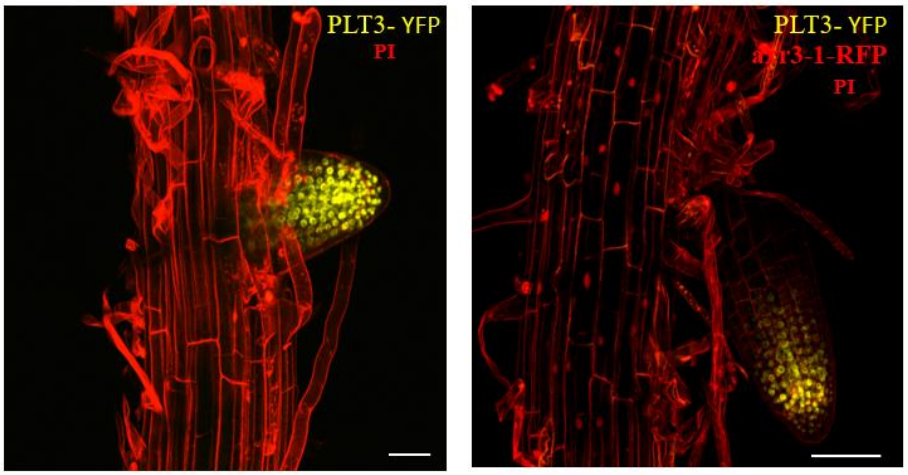

Figure34: Representative confocal images of *Arabidopsis thaliana* root expressing pPLT3::gPLT3:YFP (yellow) in case of DMSO (control) and in case of estradiol (treatment) samples were induced for 12 hours *Arabidopsis thaliana* root expressing both pPLT3::gPLT3:YFP (yellow in colour) with pG1090:XVE:: *axr3*-1:mRFP (red in colour).

3.2.5 RT-qPCR Analysis of PLETHORA Gene Expression During Inhibition of Auxin Signaling in *De Novo* Root Regeneration Assay.

The objective of the RT-qPCR analysis was to assess the gene expression levels of PLETHORA genes (PLTs), known for their significant role in *de novo* root regeneration. Previous investigations from our lab have identified PLT7 and PLT3 as key players in this process. To manipulate auxin signaling, we utilized the transgenic line pG1090:XVE::*axr3*-1-RFP and a *de novo* root regeneration assay was performed. Following the cut, induction was immediately provided by treating the leaves on 1/2 MS plate media with estradiol in it for 15 minutes. To serve as a control, DMSO was included. Sampling occurred at three distinct time points: 0 hours (pre-induction), 6 hours post-induction, and 24 hours post-induction. To ensure RNA integrity, samples were flash-frozen upon collection. Subsequently, RNA extraction was carried out, followed by cDNA synthesis using a reverse transcription kit. RT-qPCR was then performed to quantify the expression levels of PLT7 and PLT3 genes. The obtained expression data were normalized using *ACTIN 2* as a reference housekeeping gene.

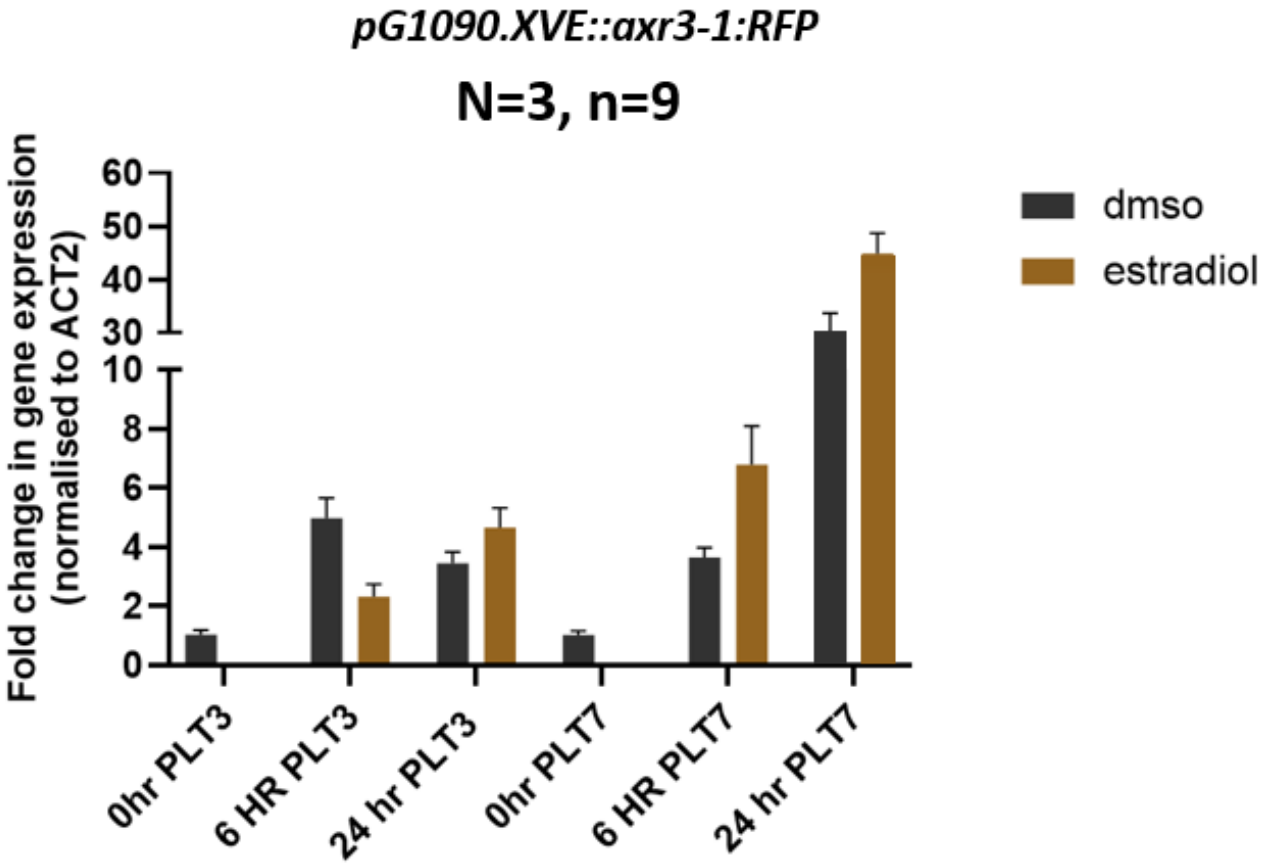

Figure35: **PLT transcript level during inhibition of auxin signaling in DNRR**. Significant upregulation of both PLT3 and PLT7 is seen at 6hr and 24hr in *axr3*-1-RFP line compare to 0hr timepoint. Expression levels were normalized to *ACTIN2*. N=1 biological replicate (7- 8 leaves), n=3 technical replicates. Error bar represent standard error mean (s.e.m).

The RT-qPCR analysis revealed a significant upregulation of the PLT7 gene at 6 hours post-induction compared to pre-induction levels. Additionally, a slight increase in PLT3 gene expression was observed at the 6-hour time point. At 24 hours post-induction, the upregulation of PLT7 gene expression was further heightened relative to the 6-hour time point, showing a remarkable 44-fold increase compared to the 0-hour time point. However, while the upregulation of PLT3 gene expression was less pronounced than that of PLT7, it still exhibited an increase compared to the 6-hour time point. These findings underscore the importance of both PLT7 and PLT3 in *de novo* root regeneration. The observed upregulation of PLT7 suggests its involvement in the response to inhibition of auxin signaling mediated by *AXR3*-1. Further investigation into the underlying mechanisms may yield valuable insights into the regulatory networks governing how this will help in *de novo* root regeneration.

3.2.6 RT-qPCR Analysis of Autophagy Gene Expression During Inhibition of Auxin Signaling in *De Novo* Root Regeneration Assay.

Recent studies from the lab have shown that *de novo* root regeneration from the leaf petiole in *Arabidopsis* is affected by disturbing the function of autophagy genes through pharmacological, genetic, and molecular perturbations (Akansha Ganguly, unpublished). This demonstrates the possibility of the existence of pathways for wound-induced regeneration for which autophagy is essential. Across eukaryotes, autophagy is a multi-step process with a sequential chain of events starting from induction, phagophore nucleation, formation of autophagosome, maturation, autophagosome fusion with lysosome, and eventual degradation and recycling of organelles and nutrients (Perrotta et al., 2020). This chain of events is coordinated by the ATG gene family, in which ATG8 proteins are the central players. ATG8 proteins are involved at all steps of autophagy, from membrane nucleation for phagophore formation to autophagosome transport and fusion to lysosomes (Nieto-Torres et al., 2021). Building upon previous data from the lab, we are here focusing on the ATG8F and ATG8H isoforms of the ATG8 protein and trying to understand how autophagy genes respond during the inhibition of auxin signaling in *de novo* root regeneration. To manipulate auxin signaling, we utilized the transgenic line pG1090:XVE::*axr3*-1-RFP and a *de novo* root regeneration assay was performed. Following the cut, induction was immediately provided by treating the leaves on 1/2 MS plate media with estradiol in it for 15 minutes. To serve as a control, DMSO was included. Sampling occurred at three distinct time points: 0 hours (pre-induction), 6 hours post-induction, and 24 hours post-induction. RT-qPCR was then performed to quantify the expression levels of ATG8F and ATG8H genes. The obtained expression data were normalized using *ACTIN 2* as a reference housekeeping gene. From the data RT-qPCR we can concluded that autophagy gene is showing some activity compare to the 0hr timepoint. There is a surge of certain subunit which again contradict the data we have during normal dnrr therefore further investigation into may yield valuable insights on how this will overall in *de novo* root regeneration or overall reprogramming

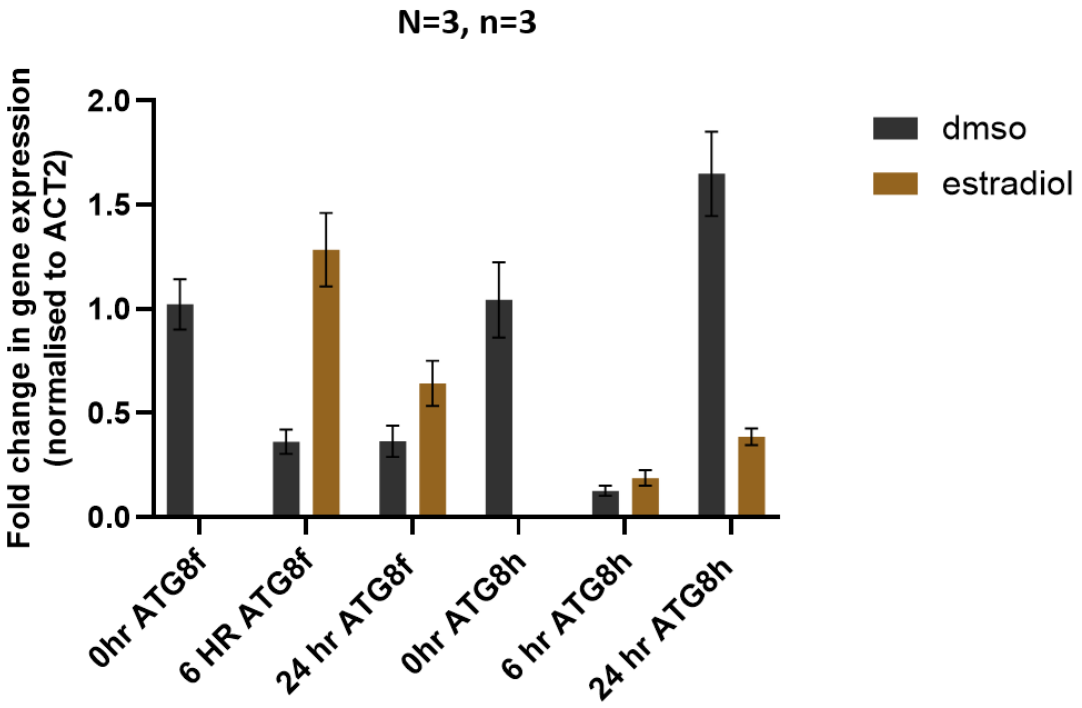

Figure 36: **ATG8 transcript level during inhibition of auxin signaling in DNRR**. upregulation of ATG8F is seen at 6hr and little downregulation of Atg8h is seen at 6hr and 24 hr in *axr3*-1-RFP line compare to 0hr timepoint. Expression levels were normalized to *ACTIN2*. N=1 biological replicate (7- 8 leaves), n=3 technical replicates. Error bar represent standard error mean (s.e.m).

3.2.7To check the expression pattern of PLT7 during inhibition of auxin signaling in DNRR.

To investigate the expression pattern of PLT7 during the inhibition of auxin signaling in *de novo* root regeneration, a touch-mediated DNRR regeneration assay was conducted using double markers (pPLT7::gPLT7:YFP with pG1090:XVE::*axr3*-1-mRFP) with a 15-minute transient induction of *axr3*-1. As *axr3*-1 serves as a negative regulator of auxin signaling, the regeneration assay revealed that a 15-minute transient induction of *axr3*-1 completely abolished *de novo* root regeneration. Expression profiles of PLT7-YFP from both markers were examined at the cut ends of 12 DPG (days post gemination) leaves at 24hr hours post-cut, 48 hours post-cut, to assess early and late responses, respectively. As a control, a DNRR regenerative assay using DMSO as a solvent control was performed to compare the expression profiles of both markers and understand how perturbation in auxin signaling alters the expression profiles of PLT7-YFP. Intriguingly, there was a notable upregulation of PLT7-YFP expression at 24hr and 48-hours post-cut (Figure B'-C') in the presence of *axr3*-1 compared to the control of 24hr and 48-hours post-cut (B-C). This suggests that inhibition of auxin signaling induces an upregulation of PLT7-YFP. However, there was a slight variation in the localization of the expression pattern in PLT7-YFP during the inhibition of auxin signaling compared to PLT7-YFP in normal settings.

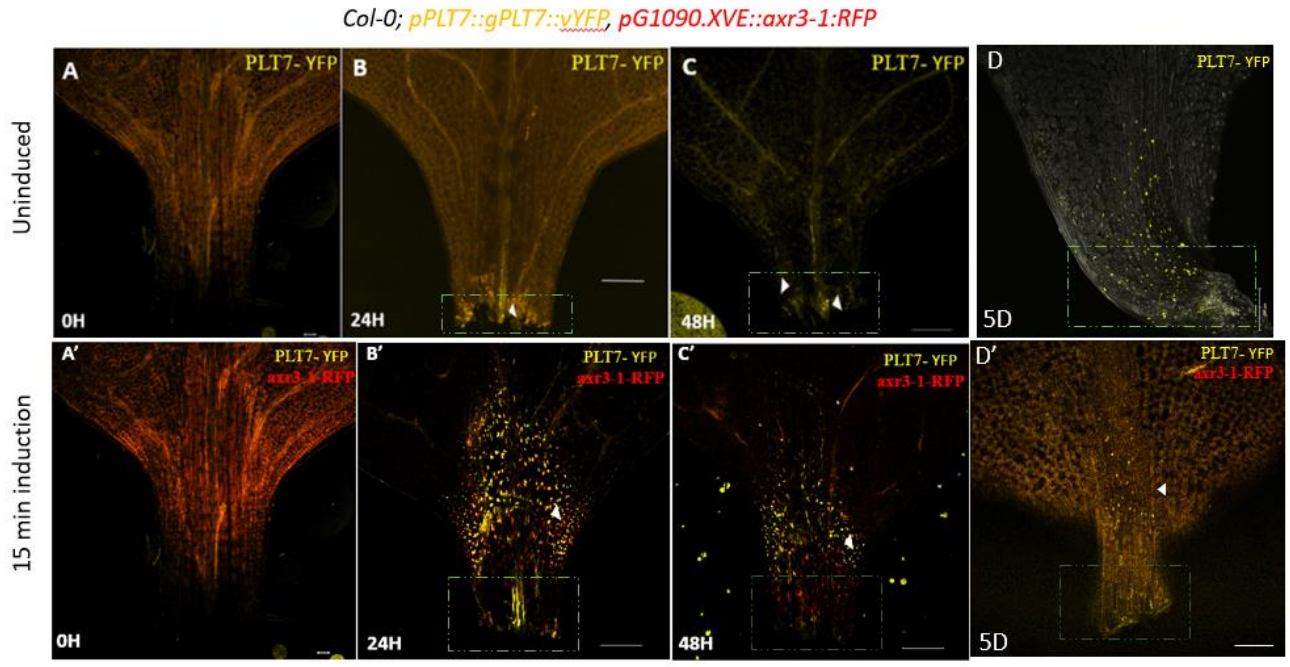

Figure37: PLT7 is highly upregulated during inhibition of auxin signaling in DNRR. (A-C) Representative confocal projections of PLT7 (yellow) expression across 24H (early response) and 48H (late response) in DNRR (with Dmso). (A'-C') PLT7 (yellow) and *axr3*-1 (red) expression at 24H and 48H in DNRR (without estradiol). Scale bar: 50µm, n=4 leaves per group.

During normal *de novo* root regeneration *PLT7* is activated in few nuclei near the cut site

during the first 24 and 48 hours which gradually increases through the petiole and eventually become systemic through the leaf during later timepoints on the other hand with *axr3-1* induction we observed a rapid surge of *PLT7* expression away from the original site of expression thus indicating proper spatiotemporal activity of PLT7 is critical to initiate dnrr not necessarily just the global expression itself. it is spatiotemporal important. From this study, it can be concluded that despite an increase in the expression level of PLT7 during the inhibition of auxin signaling, there is no regeneration during *de novo* root regeneration. The expression pattern indicates that improper localization of PLT7 near the injury sites during DNRR may contribute to the lack of regeneration.

Chapter 4 Discussion

The project aimed to undiscover the relationship between auxin signaling and PLT7 expression during *de novo* root regeneration in *Arabidopsis thaliana* provides valuable insights into the molecular mechanisms governing this complex process. By utilizing inducible promoters, we were able to manipulate auxin signaling and observe its effects on PLT7 expression and regeneration efficiency. The results of our study demonstrate a crucial role for auxin signaling in regulating *de novo* root regeneration. Inhibition of auxin signaling, achieved through overexpression of *axr3*-1, led to a drastic reduction in regeneration efficiency, indicating the essential role of auxin in initiating and sustaining the regeneration process. This observation was consistent across different induction time points, with even 15 minutes of transient induction resulting in complete abolition of *de novo* root regeneration. From this observation we can understand that at any time during *de novo* root regeneration inhibition of auxin signaling or perturbation in auxin led to completely abolishment in regeneration. From our study we also figure out that perturbation in auxin signaling is affecting severely in case of *de novo* root regeneration response but not wound healing response. These findings highlight the sensitivity of the regeneration process to perturbations in auxin signaling, emphasizing the critical importance of maintaining auxin homeostasis for successful regeneration to achieve root fate. Furthermore, our study also reveals the PLT7 expression pattern during inhibition of auxin signaling in *de novo* root regeneration. Despite the significant increase in PLT7 expression levels upon inhibition of auxin signaling, our analysis of PLT7 expression pattern revealed a distinct pattern. Rather than being localized near the injury sites, as expected during regeneration, PLT7 expression was observed to be distally localized from the petiole section. This discrepancy between PLT7 expression level and localization suggests a disruption in the normal regulatory mechanisms governing PLT7 expression during regeneration in the context of auxin signaling inhibition. our findings suggest that its interaction with transcriptional factors like PLT7 is more complexed than previously understood. Further elucidation of these regulatory networks may yield valuable insights in regeneration.

Future aspects

Understanding how auxin interacts with transcription factors like PLT7 presents an opportunity to uncover new pathways and key regulatory elements involved during *de novo* root regenreation. Our study notably reveals that inhibiting auxin signaling completely abolished *de novo* root regeneration. Interestingly, despite the increased expression of PLT7 observed following this inhibition, its localization shifts away from injury sites to the petiole section. These observations emphasize the nuanced dynamics of auxin-PLT7 interactions and stress the significance of spatial regulation in regeneration.

In the future, we aim to shed light on the influence of PLT7 on auxin biosynthesis and signaling. To do so, we've developed a PLT7 RNAi line, utilizing the RNA silencing machinery facilitated by the DICER/Argonaute complex to suppress PLT7 expression. Through thorough analysis of this RNAi line, our goal is to uncover the precise role of PLT7 in *de novo* root regeneration and its impact on auxin biosynthesis and signaling pathways. By examining how downregulation of PLT7 affects regeneration efficiency and patterns of auxin-related gene expression, we anticipate gaining a clearer understanding of the intricate relationship between PLT7 and auxin dynamics during *de novo* root regeneration. This endeavor holds promise for providing valuable insights into the molecular mechanisms governing regeneration processes and presents potential targets for manipulation to enhance regeneration efficiency in plants.

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