Evidence for the presence of *PLETHORA* homologs in plant species displaying diverse regenerative responses

A Thesis

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by

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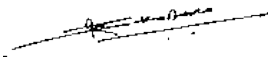
From May 2022 to Mar 2023

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled '**Evidence for the presence of *PLETHORA* homologs in plant species displaying diverse regenerative responses**' 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 **Saket Nikose** 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, teachers, friends, and anyone who has helped me to become a better person...

I born eager to explore the world,

I dreamed of doing extraordinary things,

As I set out, I quickly found that challenges abound,

With each new obstacle, my hopes came crashing down,

But, I sensed a touch amidst the darkness,

A spark of inspiration that lit a flame within my mind,

I walked the path from a wounded world,

And now, at last, I stand here fully regenerated!!!

Declaration

I hereby declare that the matter embodied in the report entitled **'Evidence for the presence of *PLETHORA* homologs in plant species displaying diverse regenerative responses'** 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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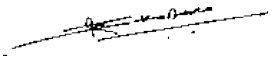

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Abstract

De novo root regeneration (DNRR) is a plant regeneration caused by mechanical injury in which the detached leaf explant from the parent plant develops roots from the cut end. The model organism *Arabidopsis thaliana* exhibits a DNRR response when the cut end of a detached leaf touches the physical surface. In contrast, if the cut end does not make contact with any physical surface, it exhibits a wound-healing response in the form of callus formation. The DNRR response in *A. thaliana* is controlled by transcription factors (TFs) such as *PLETHORAs (PLTs)*. Previous research showed that *PLTs* are essential for the DNRR. The main goal of this study is to investigate *PLT* homologs and examine similar patterns of regeneration in diverse plant species. The results demonstrate that the ability to show the DNRR response is evident in other plant species examined for the study. But nonetheless, there are certain variations also observed. Moreover, *PLT* homologous genes were identified in various plant species using a sequencing and bioinformatics approach. It is possible to show a correlation of these homologous genes in the DNRR by analyzing their gene expression patterns. Besides this, the objective is to generate polyclonal antibodies against the *Arabidopsis* PLT proteins in order to detect homologous proteins in other plant species. Finally, the ultimate objective of this research is to discover a universal regulatory module of regeneration that may be common to all plant species.

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Contributions

Contributor name	Contributor role
SN, KP, MA	Conceptualization Ideas
SN, VV, AS	Methodology
SN	Software
SN	Validation
SN	Formal analysis
SN	Investigation
BC, AK	Resources
SN	Data Curation
SN	Writing - original draft preparation
SN, VV, AS, MA	Writing - review and editing
SN	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

All organisms begin as single-celled organisms and proceed to develop into complete individuals. Animals and plants are subjected to many forms of stress and injuries from their surroundings throughout the course of their lives. To deal with stress and damage, they have the remarkable ability to regenerate missing organs or other body parts from existing tissue, cells, or body planes. In the case of animals, specialized cells help in the process of regeneration by migrating to the injured area or tissue (King and Newmark, 2012). Plants, on the other hand, have specific and robust regeneration responses that distinguish them from mammals.

Plants exhibit multiple forms of regenerative responses following organ damage or loss (Birnbaum and Alvarado, 2008). Plants can regenerate themselves from various parts of their bodies, including leaves, stems, roots, hypocotyls, and so on. "The formation of shoots and/or roots from the damaged or lost part of the organ is the main characteristic of plant regeneration."(Ikeuchi *et al.*, 2019). Cells from the injured plant can reprogram to produce a new organ or restore the identity of an existing organ. Understanding the molecular and cellular basis of regeneration is critical to know more about the factors that influence regeneration and how this knowledge could potentially be applied to agricultural sectors.

1 Regeneration in Model Organism *Arabidopsis thaliana*

A number of studies have been undertaken on the model organism *Arabidopsis thaliana*. It has a small genome, a short life span, and is easy to manage. For all of these reasons, it is an excellent choice for studying plant regeneration. Furthermore, several genetic, molecular, and computational tools facilitate an in-depth knowledge of regeneration processes.

Arabidopsis thaliana exhibits a variety of regeneration responses. It is roughly classified into two types of regeneration (Mathew and Prasad, 2021):

1.1 Tissue culture-induced regeneration- In this form of regeneration, a piece of tissue or organ (i.e., explant) is cultured on a nutrient-rich medium to regenerate the entire organism. It is also known as *de novo* organogenesis, and it can result in the production of both shoots and roots (Figure 1). It is also possible to reprogram somatic cells in tissue culture and turn them into somatic embryos, which is also known as somatic embryogenesis.

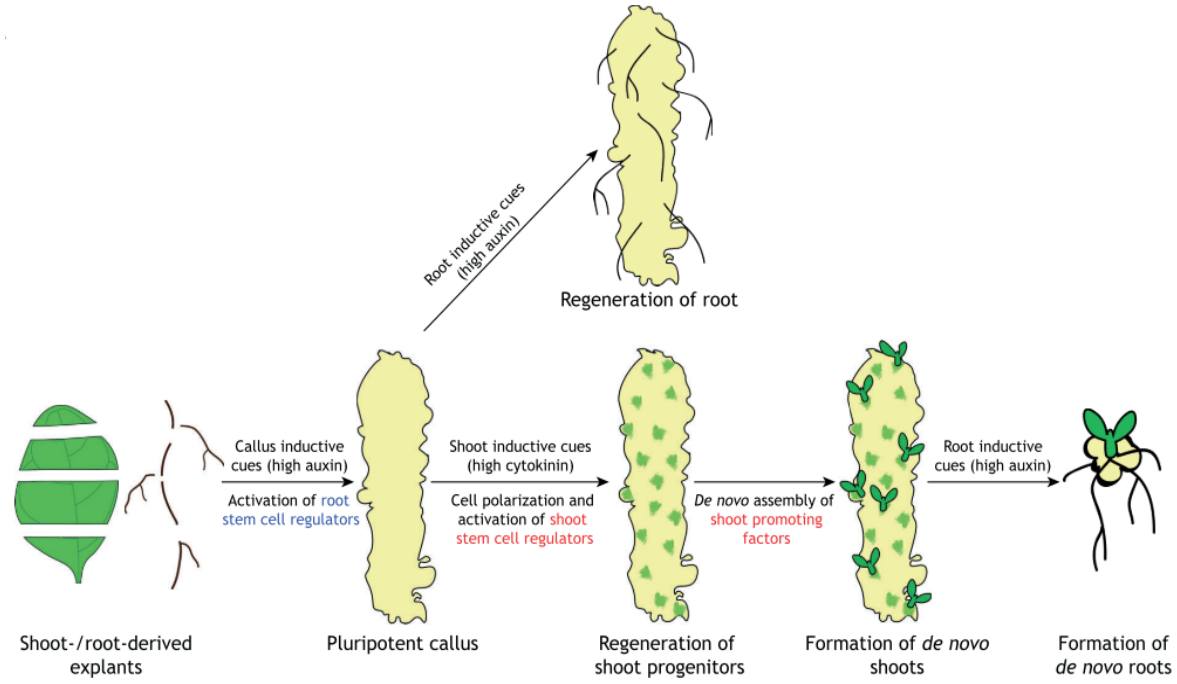


Figure 1: Tissue culture-induced regeneration. Root formation (top) and Shoot formation (bottom) in tissue culture-induced regeneration. Adapted from (Mathew and Prasad, 2021)

1.2 Mechanical injury-induced regeneration- Plants suffer damage by a variety of biotic (insects, herbivores, etc.) and abiotic elements. (heavy rains, storms, etc.). As a result, the plant should be capable of regenerating or healing the injured organ/tissue. There are two conditions in which this form of regeneration occurs. The first occurs when a wounded plant organ has a connection to the parent plant (Figure 2), whereas the second occurs when the organ is separated from the parent.

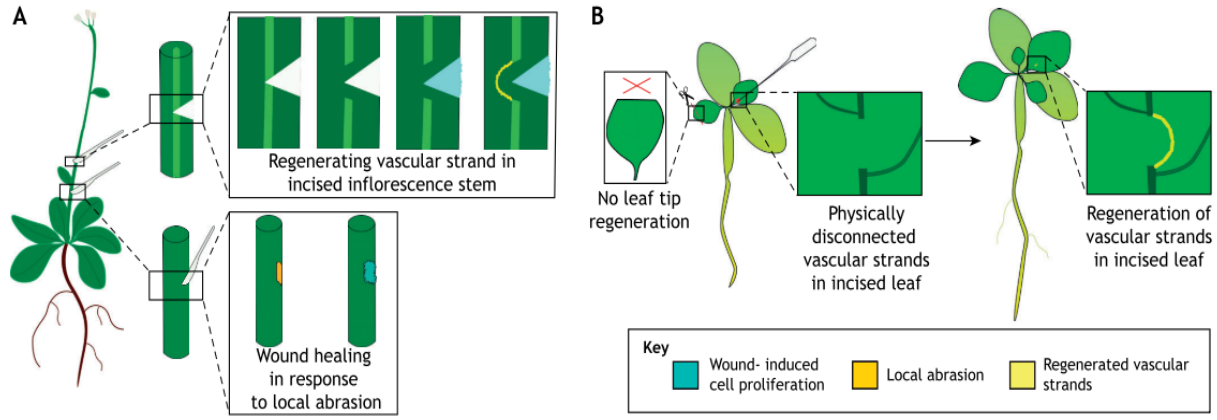


Figure.2: Mechanical injury-induced regeneration when an injured plant organ is connected to a parent. A) A stem incision interrupts the vasculature; the cells proliferate and rejoin the vascular thread. In addition, wound healing occurs in the case of a local cut. B) The incision in the leaf's midvein separates the vascular strands. The newly made loop structure eventually restores this link. Adapted from (Mathew and Prasad, 2021)

1.3 *De novo* root regeneration (DNRR)

The second form of mechanical injury-induced regeneration occurs when the organ separates from the parent plant. Studies on the model plant *Arabidopsis thaliana* have revealed that when an organ or component is removed from the parent plant, a new organ with a distinct identity from the original tissue or organ forms (Bustillo-Avendaño et al., 2018) (i.e., *de novo* organogenesis). This form of *de novo* root or shoot regeneration can occur both in natural and tissue culture conditions. (Duclercq et al., 2011, Xu and Huang, 2014)

The cut plant organs/tissue develop new roots during *de novo* root regeneration (DNRR). DNRR may occur in different plant organs. However, among these organs, the leaves exhibit a particularly remarkable response: when the leaf is cut from the plant and grown in its natural conditions, it forms new roots from the cut side of the leaf (Figure 3) (Shanmukhan *et al.*, 2021).

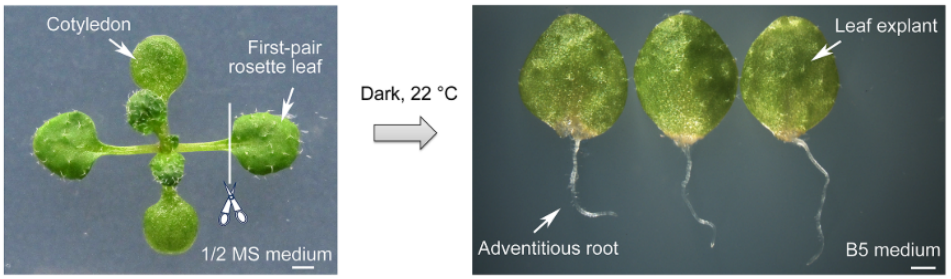


Figure 3: *De novo* root regeneration in the model plant *Arabidopsis thaliana* (Wild Type). The growth medium is hormone free. Reproduced from (Chen *et al.*, 2014).

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). This type of regeneration approach can be distinguished by the fact that it is not dependent on the exogenous hormones found in the culture medium for tissue-culture-mediated regeneration. 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 Touch-dependent nature of the DNRR

In the hormone-free media, the DNRR response occurs by the cut end of *Arabidopsis* leaves. Nonetheless, the cut end of *Arabidopsis* leaves leads to another response, that of wound healing in the form of callus formation (Shanmukhan et al.,

2021). What determines regeneration fate (*de novo* root vs. callus) is the question here. The physical contact of the cut end with the surface of a solid or liquid medium influences the decision to make the DNRR over the callus. When the leaves are positioned such that the cut end meets the hormone-free media surface, the leaf explant produces de novo roots (Shanmukhan *et al.*, 2021). On the other hand, when the leaves were placed in a way that the cut end did not touch the media (i.e., in the air), they showed a wound-healing response in the form of callus formation (Figure 4). The authors additionally looked at the auxin level of the cut leaf explant. The auxin level is higher in the leaf where the cut section comes into contact with the agar medium than in the leaf where the cut section does not come into contact with the agar media surface (Shanmukhan *et al.*, 2021). This demonstrates that auxin has a role in the DNRR response.

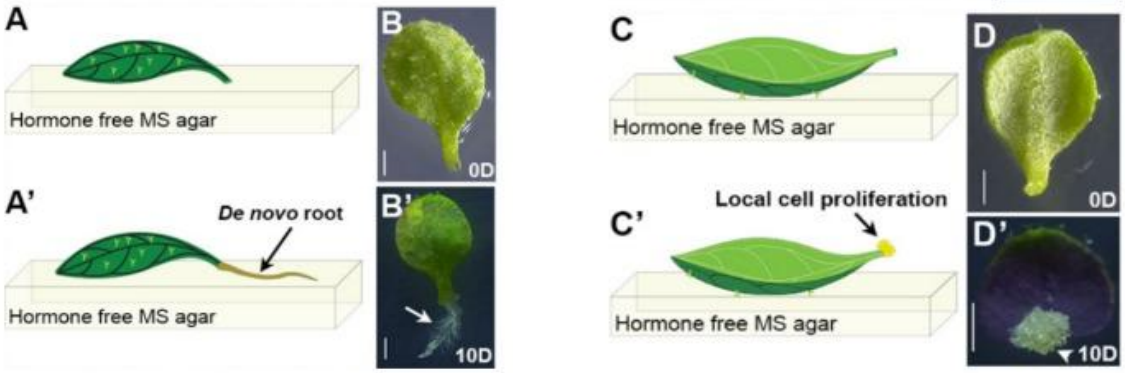


Figure.4: Regenerative response at the cut end in the *A. thaliana*. DNRR response from the cut part of a leaf in the presence of touch (A, A', B, B'). And the formation of callus when the leaf is placed on the MS-agar media so that the cut part does not touch media(C, C', D, D'). Adapted from (Shanmukhan *et al.*, 2021).

1.3.2 Cellular and Molecular Foundation of DNRR

A) Cellular Basis- A cell must repair and initiate a survival strategy in the *de novo* organogenesis process Here, the cells must reprogramme themselves in order to take on the identity of the new organ. To do this, the initial cells capable of becoming regeneration-competent cells must undergo dedifferentiation in order to achieve the stem cell-like fate. Then they may transform into cells with a distinct identity (here, root cells) from the parent one. Adult stem cells from aerial organ vascular tissue, such as procambium or cambium cells, promote the emergence of new roots (da Rocha Correa et al., 2012; Chen et al., 2016). Following cellular dedifferentiation and competency, the cells must undergo proliferation, which is required for de novo organogenesis. Finally, the cells must acquire a new cell fate, which is achieved by hormone responses and cell type-specific gene regulation.

B) Molecular Basis- The phytohormones auxin and cytokinin are critical in determining root or shoot fate during regeneration. If the ratio of auxin to cytokinin is high, it will result in root formation, while if high cytokinin is present as compared to auxin, then it will lead to shoot formation (Ikeuchi *et al.*, 2019). Auxin is crucial for the DNRR response (Pop *et al.*, 2011; Xu, 2018; Mathew and Prasad, 2021). From the studies (Sun *et al.*, 2016), It is clear that when the explant is cultivated in conditions containing auxin biosynthesis inhibitor or auxin polar transport inhibitor, root regeneration does not happen. PIN-FORMED 1 (PIN1), PIN2, PIN3, and AUXIN RESISTANT 1 (AUX1) are auxin transporters involved in DNRR development (Braybrook and Harada, 2008). Auxin accumulation also activates factors such as AUXIN RESPONSE FACTOR (ARFs) and WUSCHEL- RELATED HOMEOBOX (WOX11, WOX12, WOX5, and WOX7) that help cells to acquire new fate (Liu et al., 2014; Chen et al., 2016). Hence, the auxin polar transport and biosynthesis are indispensable for DNRR response.

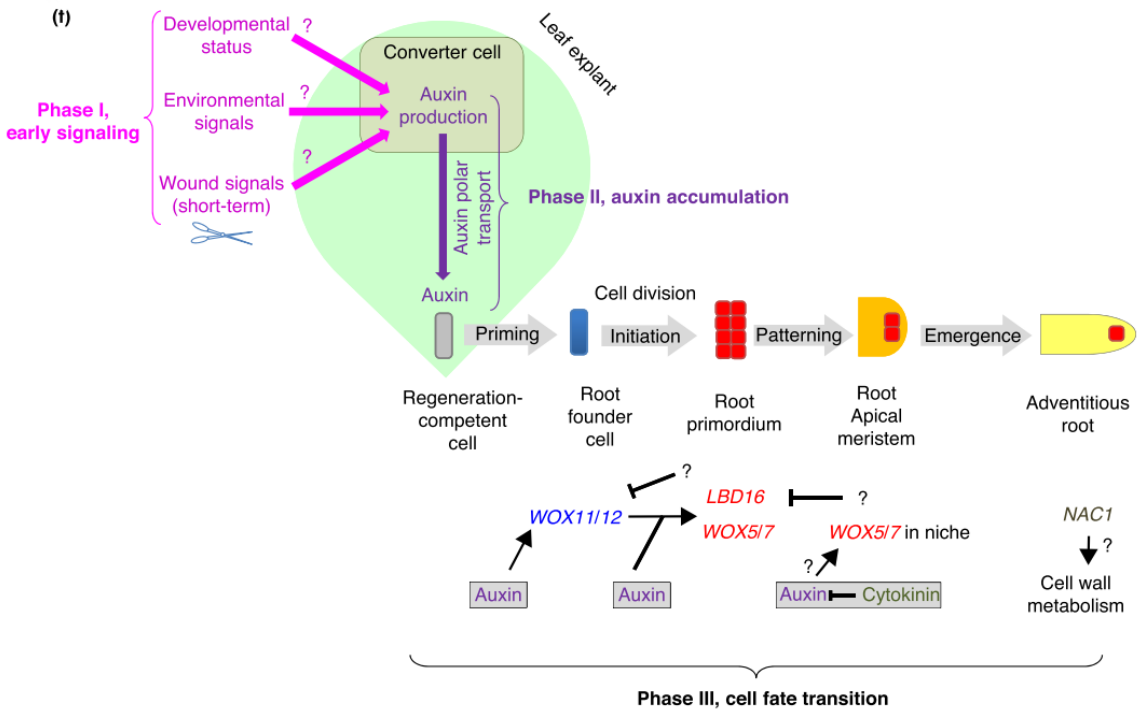


Figure 5: Cellular and Molecular framework of DNRR. Adapted from (Xu, 2018)

C) Three phases of the DNRR- Author Lin Xu, 2018 described the cellular and molecular framework of the DNRR in three phases (Figure 5). The first phase consists of early signaling, which is mediated by wounding. The detached explant can sense different cues, such as environmental and wound signals. There are additional short-term as well as long-term wound cues that may play a role in the

transmission of signals from the site of the wound to the converter cells (The cells that take early signals as input and produce auxin as output) (Xu, 2018). The second phase is where the accumulation of the auxin happens. Auxin accumulates in the cambium and procambium cells which reprogram and give rise to new roots. The last Phase has to do with a change of cell fate. This phase is divided further into four parts. The first stage involves the conversion of regeneration-competent cells into root founder cells. Root founder cells divide to generate the root primordium in the second stage. Root primordium cells emerge in the third stage, giving birth to the root apical meristem. Finally, at the end of the process, the root tip emerges from the meristem cell layer. (Figure 5).

2 Motivation for the project

After discussing the above mechanisms of the DNRR. There are a lot of unanswered questions. First of all, why does a detached leaf explant form the *de novo* root? How does the detached leaf sense the physical surface? What are the factors that control the touch-dependent nature of the DNRR?

Previous research has indicated that essential transcription factors (TFs) like as *PLT3*, *PLT5*, and *PLT7* play a vital role in plant regeneration, as in the case of DNRR (Kareem *et al.*, 2015; Radhakrishnan *et al.*, 2020; Shanmukhan *et al.*, 2021). *PLT7* expression is higher when the cut end of the leaf explant encounters the growth media surface (i.e., With Touch- WT) than when the cut end does not touch the growth media surface (i.e., Without Touch- WOT) (Figure 6). A similar expression pattern is shown by the *PLT3* and *PLT5* genes (Shanmukhan *et al.*, 2021).

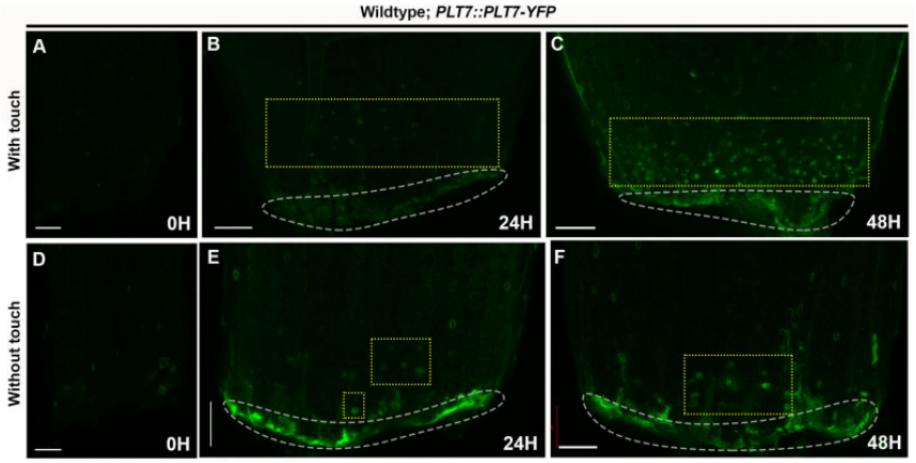


Figure 6: Expression of the *PLT7* gene in With Touch (WT) and in Without Touch (WOT). The number of cells expressing the *PLT7* signal is more in the WT (A, B, C) as compared to the WOT (D, E, F). H: hours post cut, Adapted from (Shanmukhan *et al.*, 2021).

Interestingly, when the *PLT7* is overexpressed in the leaf explant without touching the media, it shows a DNRR response (Figure 7). This finding suggests that *PLT7* is sufficient to induce the DNRR and can overcome the need for physical contact with the surface. Considering all the results from the above study, it is clear that *PLETHORA (PLT)* genes are essential for the DNRR.

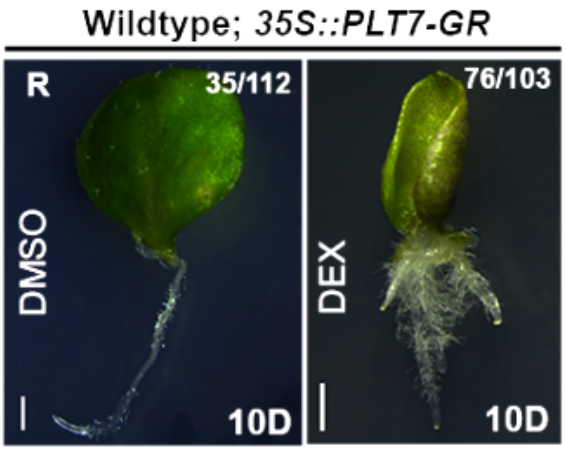


Figure 7: Overexpression of the *PLETHORA 7 (PLT7)* results in the multiple roots in the Without touch leaf sample. D: days post cut, Adapted from (Shanmukhan *et al.*, 2021).

This *de novo* root organogenesis phenomenon is also shown by other plants (Chen *et al.*, 2014), and this regeneration phenotype might be conserved across the dicot plants (Mhimdi and Pérez-Pérez, 2020). However, do other plants show the touch-dependent nature of the DNRR similar to *Arabidopsis thaliana*? If they show the DNRR response, what is the cellular and molecular mechanism behind this? Moreover, are the factors responsible for DNRR (such as *PLETHORAs*) conserved across the plant species, or do they play any role in the DNRR response?

3 Goal of the project

The primary objective of this study is to explore *de novo* root organogenesis (DNRR) in different plant species. Moreover, to discover the possible factors controlling root regeneration to see if they are conserved across these plant species.

Aim 1- To investigate the variation in regenerative responses across plant species taken from the IISER PUNE campus.

Objective 1.1- To perform a DNRR assay similar to *Arabidopsis thaliana* to check the regeneration response of different plant species.

Aim 2- To check for the *PLETHORA (PLTs)* homologous genes in different plant species and their role in the DNRR.

Objective 2.1- To amplify the *PLT* genes, use sequencing methods to sequence them, then do analysis using bioinformatics tools to determine sequence homology.

Objective 2.2- To compare the gene expression profile of the *PLT* genes with the help of semi-quantitative PCR to show a correlation with the *de novo* root regeneration.

Aim 3- To detect PLT7 homologous protein in different plant species.

Objective 3.1- To raise a polyclonal antibody against *Arabidopsis thaliana* PLT7 protein and use this antibody to detect the PLT7 proteins in the different plant species.

Chapter 2 Materials and Methods

2.1 Plant Species

All of the plants that are under consideration for the research project are of the wild type and were collected from the Green House area at the IISER PUNE campus, Maharashtra, India. All the species from the above-selected region are angiosperms from various genera and families. A total of 28 plant species belonging to 20 families were screened (Table 1). The plant species were selected randomly from the given location to screen for the regeneration assay.

Sr. No.	Name of the plant	Family
1.	*Impatiens walleriana (Iw)*	*Balsaminaceae*
2.	*Launaea nudicaulis (Ln)*	*Asteraceae/Compositae*
3.	*Centratherum punctatum (Cp)*	
4.	*Sphagneticola trilobata (Sp)*	
5.	*Chrysanthemum indicum (Ci)*	
6.	*Sonchus (So)*	
7.	*Mentha × rotundifolia (Mr)*	*Lamiaceae*
8.	*Plectranthus scutellarioides (Ps)*	
9.	*Ocimum tenuiflorum (Ot)*	
10.	*Pseuderanthemum carruthersii (Pc)*	*Acanthaceae*
11.	*Buddleja asiatica (Ba)*	*Scrophulariaceae*
12.	*Peperomia obtusifolia (Po)*	*Piperaceae*
13.	*Ficus spp. (Fs)*	*Moraceae*
14.	*Trophis scandens (Ts)*	
15.	*Euphorbia heterophylla (Eh)*	*Euphorbiaceae*
16.	*Oxalis (Ox)*	*Oxalidaceae*
17.	*Cholorophytum comosum (Cco)*	*Asparagaceae*
18.	*Maerua angolensis (Ma)*	*Capparaceae*
19.	*Geranium aralia (Ga)*	*Araliaceae*
20.	*Dendropanax arboreus (Da)*	

21.	*Pelargonium hortorum (Ph)*	*Geraniaceae*
22.	*Malva sylvestris (Ms)*	*Malvaceae*
23.	*Lecointea amazonica (La)*	*Leguminosae*
24.	*Capsicum frutescens (Cf)*	*Solanaceae*
25.	*Cuphea carthagenesis (Cca)*	*Lythraceae*
26.	*Epiphyllum oxypetallum (Eo)*	*Cactaceae*
27.	*Licania membranacea (Lm)*	*Chrysobalanaceae*
28.	*Azadirachta (Az)*	*Meliaceae*

Table-1: List of plant species screened for the study according to the scientific names (short name) and family names.

2.2 Growth conditions

All of the plant species considered for the study were cultivated in a greenhouse environment with 24/7 white light conditions at a temperature of 22 ± 1 °C.

2.3 *De novo* root regeneration assay

The growth medium used for the experiment is a hormone-free soil mixture. The soil mixture contains, Soil, Vermiculite, and Soilirite in a 2:1:1 ratio. The Murashige & Skoog medium (MS) [with CaCl2 and vitamins]- 4.4 grams/liter (Himedia, Ref-PT021-1L) was added to the soil mixture. To study the DNRR response in the disconnected leaves, the mature plant leaves of the same age were excised using scissors and kept on the hormone-free soil mixture. The regeneration response was scored on different days depending on when the plant responded.

2.4 RNA extraction and cDNA synthesis

The DNRR leaf petiole samples were taken at different intervals in time (0 DPC, 5 DPC, 6 DPC, 7 DPC, and 8 DPC) (DPC- days post cut) and snapped-frozen in liquid N2 and preserved at - 80 degrees Celsius for the extraction of RNA. TRIzol reagent was used to extract RNA from the petiole of a leaf sample. After plant tissue homogenization, it was dissolved in the TRIzol reagent. Then, Chloroform was added in the 1:5 ratio (TRIzol: Chloroform). The RNA was precipitated using 8M Lithium Chloride (LiCl) and isopropanol. 80% ethanol was used for washing the resultant RNA pellet. Finally, the RNA was dissolved in DEPC water and measured with a Nanodrop for subsequent use. The Takara Primescript RT reagent Kit (Cat. # RR037A) was used to prepare cDNA from the isolated RNA.

2.5 Degenerate primers designing

To amplify the gene of interest for which the sequence is not available, the degenerate primers were designed. First, via the NCBI database (https://www.ncbi.nlm.nih.gov/), the available PLETHORA (PLTs) protein sequences of the same family of the plant species chosen for the study were assembled. Then, using the program Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), multiple sequence alignment (MSA) was done to identify conserved regions among homologous protein sequences. Then the conserved amino acid (a.a.) sequence region was reverse-translated into the corresponding nucleotide sequence using the available gene sequence of the same protein. The degeneracy of the nucleotide sequence is identified from the conserved nucleotide regions, and the primers were designed (Table 2) (using Snapgene software- https://www.snapgene.com/) to match more of the number of hits, keeping non-specificity in mind that arises due to high degeneracy (See Results Section 3.2.1). The expected amplicon size of the *PLT* gene from cDNA is approximately 100-150 bps. The workflow of the degenerate primer designing is summarised in (Figure 8).

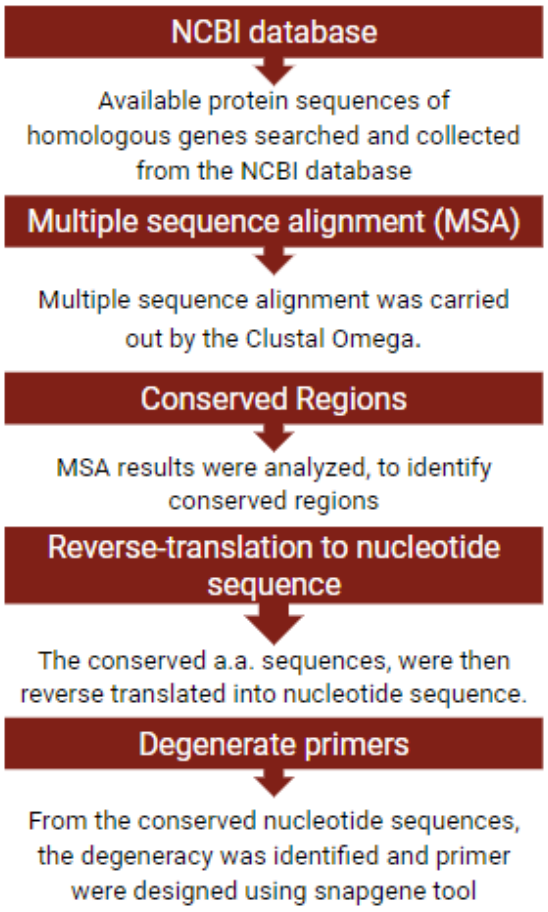


Figure 8: Degenerate primer designing pipeline

Sr. No.	Gene Primer	FP	RP
1.	At_PLT7	5'-CATCTATGGGATAACAGCTGTAG-3'	5'-ATTTTAAAGCTGCCAAGTCATA-3'
2.	Ss_PLT7	5'-CATCTATGGGATAACAGCTGTAG-3'	5'-GAGAGCGGCCAAATCATA-3'
3.	EcPLT7	5'-CATCTTTGGGATAATAGCTGTAG-3'	5'-ACTTCAAGGCAGCCAAATCATA-3'
4.	StPLT7	5'-CATCTATGGGATAACAGCTGTAG-3'	5'-TTTAGAGCTGCCAAGTCATA-3'
5.	Comm_PLT7	5'-GGACAAAGAACTTCAATTTATCG-3'	5'-AAGTACACTTGACGTCCTTTTC-3'

Table-2: List of degenerate primer designed for *PLT7* gene

2.6 PCR and Semi-quantitative PCR

To determine the biological activity of the *PLT* genes in several plant species, degenerate primers were designed, and PCR (Takara PrimeSTAR GXL DNA Polymerase, Cat. # R050A) was performed. The gradient temperature PCR (Eppendorf Mastercycler X50s) tried to amplify (amplicon size: 100-150 bps) the homologous gene in different plants using the forward and reverse primers combinations. The semi-quantitative was performed at 30X cycles to compare the gene expression at different time points. Standard techniques were used for separating the PCR products on 2% agarose gels and visualizing them following ethidium bromide staining.

2.7 Sequencing of amplicon and analysis results

The PCR amplicon is purified QIAGEN PCR purification Kit (Cat. # 28106) and sent to company BARCODE BIOSCIENCES (BBS) for sequencing. The sequencing results were analyzed with the help of the software DNASTAR Navigator 17 (https://www.dnastar.com/) and EMBOSS needle (EMBOSS Needle < Pairwise Sequence Alignment < EMBL-EBI). The *Arabidopsis* Information Resource (TAIR) database (TAIR- Home Page) and BLAST tool were used to get the gene sequence of *Arabidopsis thaliana*.

2.8 Expression and Induction of PLT7 protein

The *PLT7* CDS sequence was cloned in the pET28a vector (N-terminal 6xHis-tag) expression vector using BamHI and HindIII restriction sites, and protein expression

was done in Rosetta (DE3) strain. Two types of clones were generated to express full-length PLT7 protein (using Rosetta-pET28a.cPLT7) and PLT7 protein peptide containing two AP2/ERF domains (using Rosetta-pET28a.cPLT7.Domain). The primary culture was maintained at 37 °C in order to attain an OD between 0.6-0.8. After desired OD, the PLT7 full-length protein was induced at 37℃ for 6hrs with an IPTG concentration of 0.7mM, while the PLT7 AP2/ERF domain-specific peptide was induced at 30℃ for 6rhs with an IPTG concentration of 0.2mM.

2.9 Protein purification

The full-length PLT7 protein and AP2/ERF domain protein were purified in the denatured state. For the purification of both full-length protein and protein peptide from the bacteria, the cell pellets were collected using the centrifuge and lysed with the help of sonication in the Urea lysis buffer containing 8M Urea, 10mM NaH2PO4, 10mM imidazole, 10mM tris (pH8), and dH2O. The lysate was cleared by centrifuging at 12,000 rpm for 50 mins at 4°C. The proteins were purified with the help of the Ni-NTA agarose beads in batch purification. The Ni-NTA resin was first charged with Ni2+ by washing with a 100 mM NiCl2 solution. At 4°C, the beads were incubated with the supernatant for 1.5-2 hours. The beads were washed in a buffer that included 20 mM imidazole for the full-length protein and 10 mM imidazole for the domain peptide. Both proteins were eluted at a single step in 250 mM of imidazole. The purified proteins were stored at -80°C.

2.10 SDS-PAGE

SDS-PAGE was used to examine the protein preparation. 12% resolving gel (Bis-Acrylamide, 1.5M Tris pH 8.8, 10% SDS, 10% APS, TEMED, dH2O) and 6% stacking gel (Bis-Acrylamide, 0.5M Tris pH 6.8, 10% SDS, 10% APS, TEMED, dH2O) were used to separate the proteins. The protein sample was loaded with the 5X Laemmli Buffer (0.5M Tris-HCl ph 6.8, 0.25% BPB, SDS, Glycerol, Beta-mercaptoethanol) and heated at 95°C for 10 minutes at 800 rpm. The gel was loaded with samples and run at 90-120V. After running the gel, it was then stained for an hour with coomassie blue, followed by overnight destaining.

2.11 Microscopy and PCR gel/SDS PAGE documentation

The Leica S8 APO stereo zoom microscope was used to document the DNRR samples. While the PCR gels and SDS-PAGE gels were documented on the Syngene G-box gel doc system.

2.12 Image analysis

The gel images for semi-quantitative PCR are analyzed using the Fiji program (Schindelin *et al.*, 2012). Furthermore, the data is plotted in GraphPad Prism version 9.5.1 software (www.graphpad.com).

Chapter 3 Results

Section 3.1 Regenerative responses across plant species

3.1.1 Screening for the *De novo* root regeneration (DNRR) response.

All the selected plant species were screened for the *de novo* root regeneration (DNRR) response, where the cut end of the leaf was kept on the growth medium (WT- With Touch group). Most of the plants showed similar responses to the model plant *A. thaliana*, i.e., *de novo* root formation (DNRR) (17 plant species) (Figures 9 and 10). A few of them (2 plant species) showed first callus formation and then root formation (Figure 9). While some showed wound healing (3 plant species) and callus formation (6 plant species) response (Figure 9). So, out of a total number of plant species, around 60% of them show a DNRR response. Furthermore, only 13 of the 17 DNRR plant species were chosen for the DNRR study based on their regeneration efficiency (number of samples showing the response out of the total number of samples) and convenience of plant sample handling (Table 3).

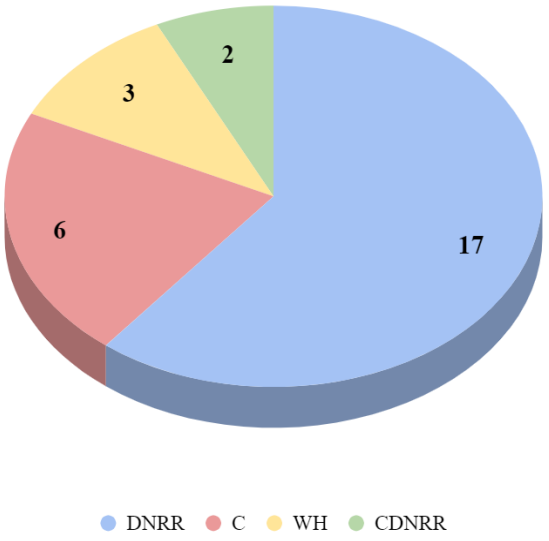


Figure 9: Regeneration response of all the plant species (28) screened for the DNRR response. The leaves of all the plant species were excised from the plant, and the cut end of the leaf kept touching the growth medium. DNRR- *De novo* root regeneration response, C-Callus formation response, WH-wound healing response, CDNRR- callus followed by DNRR response. Each experiment was performed with two biological replicates, and each replicate contained 30 leaves.

Sr. No.	Name of the plant	Family
1.	*Impatiens walleriana (Iw)*	*Balsaminaceae*
2.	*Launaea nudicaulis (Ln)*	*Asteraceae/Compositae*
3	*Centratherum punctatum (Cp)*	
4.	*Sphagneticola trilobata (Sp)*	
5.	*Chrysanthemum indicum (Ci)*	
6.	*Sonchus (So)*	
7	*Mentha × rotundifolia (Mr)*	*Lamiaceae*
8.	*Plectranthus scutellarioides (Ps)*	
9.	*Pseuderanthemum carruthersii (Pc)*	*Acanthaceae*
10.	*Buddleja asiatica (Ba)*	*Scrophulariaceae*
11.	*Peperomia obtusifolia (Po)*	*Piperaceae*
12.	*Ficus spp. (Fs)*	*Moraceae*
13.	*Euphorbia heterophylla (Eh)*	*Euphorbiaceae*

Table-3: Final list of plant species (total-13) considered for the DNRR study according to their scientific names (short name) and family names. Some plants belong to the same family (*Asteraceae/Compositae and Lamiaceae*). When the detached leaves of the above plant species come into contact with the surface of the growing media, they all display a DNRR response.

3.1.2 The *de novo* root organogenesis (DNRR) is conserved across plant species screened.

To investigate the *de novo* root regeneration (DNRR) response in the plant species, a regeneration assay similar to *Arabidopsis thaliana* was performed (Shanmukhan *et al.*, 2021) (Figure 4). The mature plant leaves were cut and placed abaxially side down (WT, i.e., With Touch, test group) such that the cut end of the leaf made touch with the growing media (i.e., Soil added with MS water). They were also positioned in another orientation to keep the adaxial side down (WOT, i.e., Without Touch, also called a control group).

All plant species in the WT group show *de novo* roots from the detached leaf (Figure 10), a response comparable to that of *Arabidopsis thaliana* (Figure 4). Some plants developed single *de novo* roots (e.g., *Impatiens walleriana, Launaea nudicaulis*), but the majority of them had multiple roots from the cut end. (e.g., *Plectranthus scutellarioides, Chrysanthemum indicum, Peperomia obtusifolia,* etc.) (Figure 10).

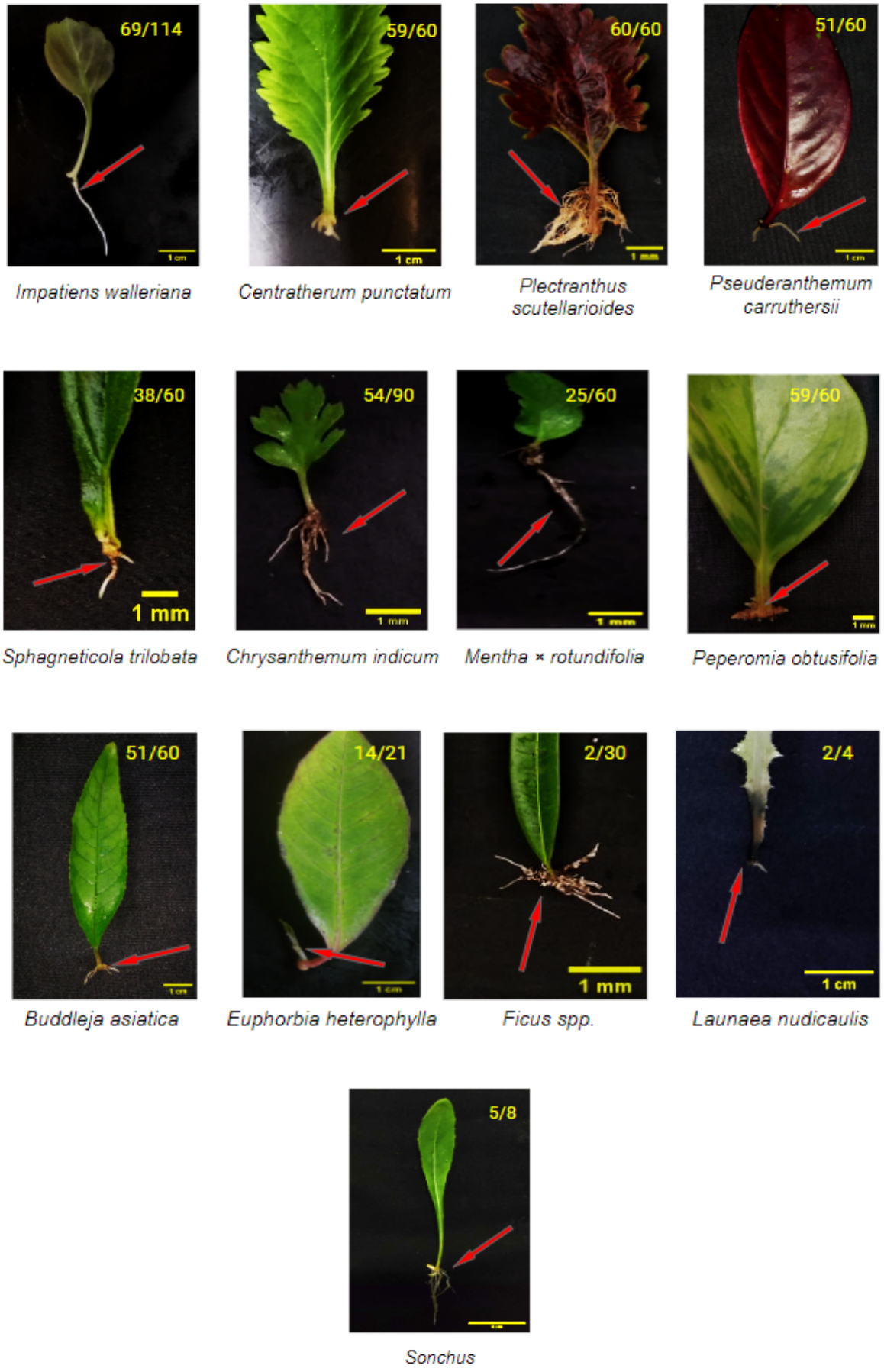
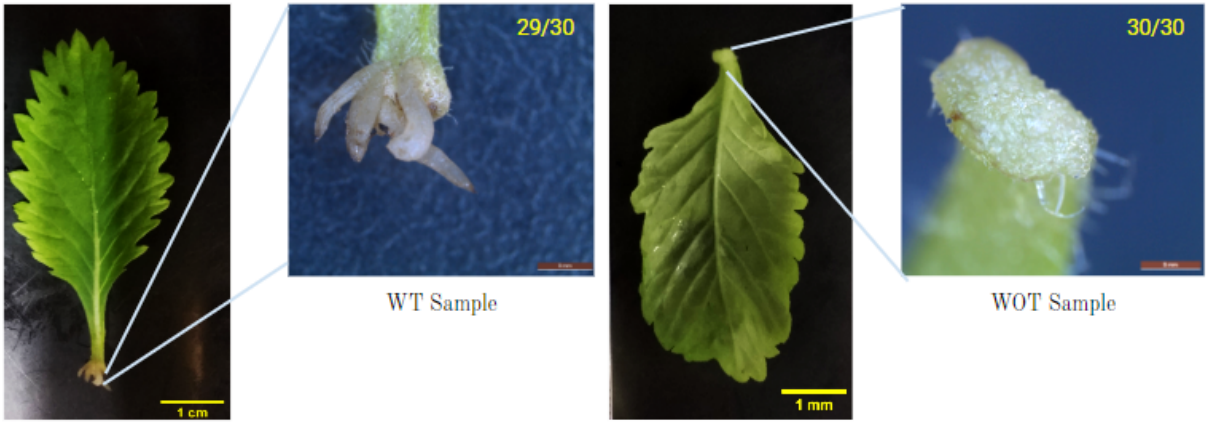


Figure 10: Regeneration response in the test group (i.e., With Touch- WT samples). All the plant leaves (13 species) produced *de novo* roots from the detached leaves. The numbers show the regeneration frequency (i.e., Number of samples showing response/Total number of samples).

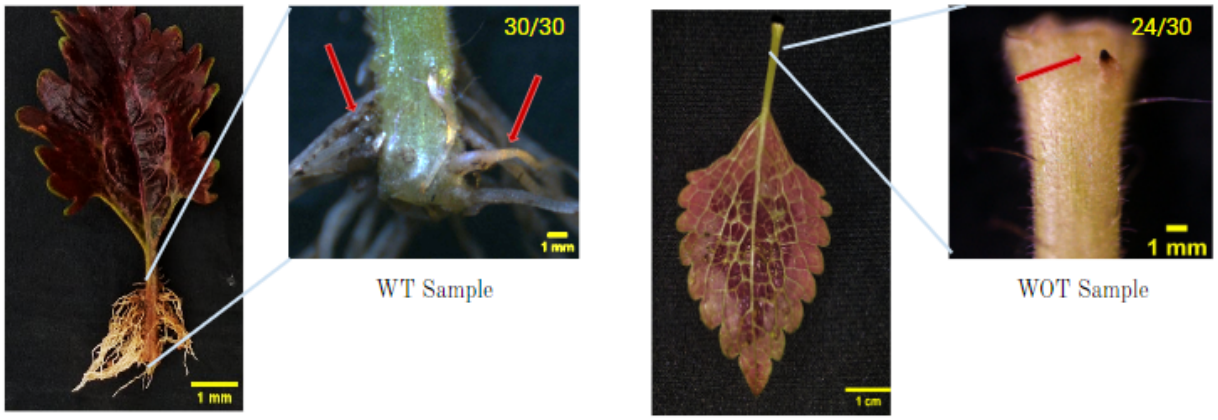
3.1.3 All plants do not require touch to show DNRR response.

The regeneration response was different in the WOT group, where the cut end did not touch the growing media, compared to the WT group. (Figure 11). Not all of the plants displayed callus or wound healing like *Arabidopsis thaliana* (Figure 4). Six plants showed DNRR in both the WT and WOT groups (naturally touch-independent). (Table-4). Seven plants' responses were identical to Arabidopsis thaliana's touch-dependent nature (DNRR in WT and wound healing response or callus production WOT) (Table-4). Figure 12 shows a touch-dependent plant (*Centratherum punctatum-Cp*) compared to a touch-independent plant. (*Plectranthus scutellarioides- Pc*). Plant *Cp* has roots in WT and callus in WOT groups, whereas plant Pc has roots in both WT and WOT groups.

Figure 11: Regeneration response in the control group (i.e., Without Touch- WOT samples). Some plant leaves produce *de novo* roots from the cut end, while others show callus or wound healing responses. The numbers show the regeneration frequency (i.e., Number of samples showing response/Total number of samples).



A) Touch-dependent plant: *Centratherum punctatum (Cp)*



B) Touch-independent plant: *Plectranthus scutellarioides (Pc)*

Figure 12: Comparison of a touch-dependent and touch-independent plant. A) In the touch-dependent plant *Centratherum punctatum*, in the WT response detected leaf shows the *de novo* roots, and in the WOT response, it shows the callus formation. B) In the touch-independent plant *Plectranthus scutellarioides,* the detached leaf shows *de novo* roots in both WT and WOT response. The numbers show the regeneration frequency (i.e., Number of samples showing response/Total number of samples).

A) Touch-dependent plants:

Sr. No.	Name of plant	Family	WT	WOT/Control
1.	*Centratherum punctatum*	*Asteraceae/Compositae*	Root	Callus
2.	*Sphagneticola trilobata*		Root	WH
3.	*Sonchus*		Root	WH
4.	*Mentha × rotundifolia*	*Lamiaceae*	Root	Callus
5.	*Buddleja asiatica*	*Scrophulariaceae*	Root	WH/Callus
6.	*Ficus spp.*	*Moraceae*	Root	WH
7.	*Euphorbia heterophylla*	*Euphorbiaceae*	Root	WH/Callus

B) Touch-independent plants:

Sr. No.	Name of plant	Family	WT	WOT/Control
1.	*Impatiens walleriana*	*Balsaminaceae*	Root	Root/Callus
2.	*Chrysanthemum indicum*	*Asteraceae/Compositae*	Root	Root
3.	*Plectranthus scutellarioides*	*Lamiaceae*	Root	Root
4.	*Pseuderanthemum carruthersii*	*Acanthaceae*	Root	Root
5.	*Peperomia obtusifolia*	*Piperaceae*	Root	Root
6.	*Launaea nudicaulis*	*Asteraceae/Compositae*	Root	Root

Table-4: Classification of plants based on the touch-dependent nature of the *de novo* root organogenesis. Group (A) Touch-dependent plants, where detached leaf shows root in With Touch (WT) response and Callus/Wound healing (WH) in the Without Touch (WOT) response. Group (B) Touch-independent plants, where the detached leaf shows Root in both With Touch (WT) as well as Without Touch (WOT) response.

3.1.4 All the DNRR Plants belong to diverse genera and families.

A phylogenetic tree at the family level was created to assess the diversity of the chosen plant species (Figure 13). All of the plant species are members of the group *Mesangiospermae*, suggesting that they are all angiosperms. The phylogenetic tree shows that the plant species considered for the study are diverse. Some are distantly related to the model plant *Arabidopsis thaliana*'s family *Brassicaceae*, while others are closely related. Early diversification occurred in the *Piperaceae* plant family. *Brassicaceae*, *Moraceae*, and *Euphorbiaceae* are all related families.

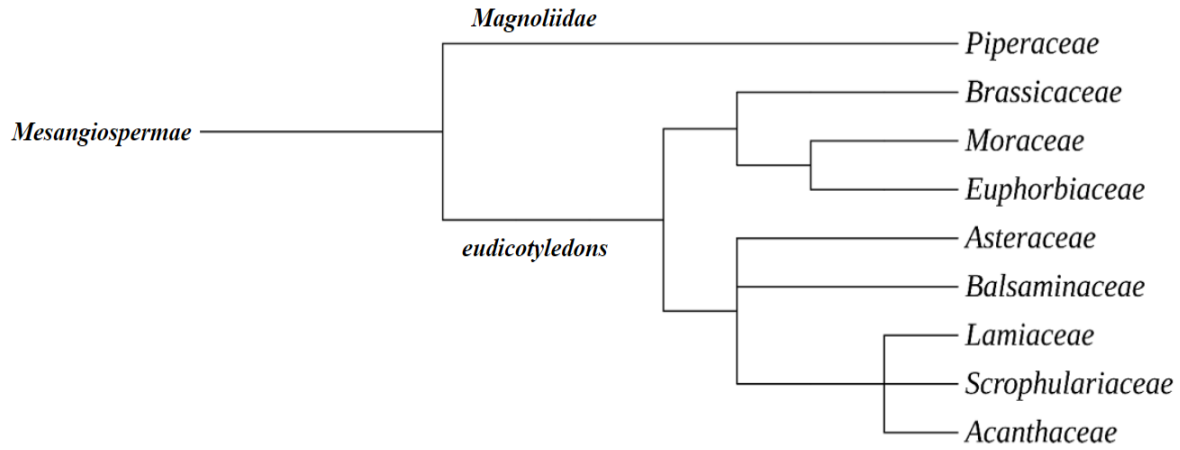


Figure 13: Phylogenetic tree of plant species considered for the DNRR study (Including *Arabidopsis thaliana* family *Brassicaceae*). The phylogenetic tree was constructed at the family level with the help of the online software Phylot (https://phylot.biobyte.de/). The *Piperaceae* family was considered as an outgroup.

Sr. No.	Name of plant	WT (DPC)	WOT/Control (DPC)
1	*Impatiens walleriana (Iw)*	6	9-11
2	*Mentha × rotundifolia (Mr)*	6	6
3	*Centratherum punctatum (Cp)*	7	7
4	*Plectranthus scutellarioides (Ps)*	7	11
5	*Sonchus (So)*	7	7
6	*Chrysanthemum indicum (Ci)*	8	10-11
7	*Buddleja asiatica (Ba)*	9	9
8	*Peperomia obtusifolia (Po)*	12	12-14
9	*Pseuderanthemum carruthersii (Pc)*	14	17-20
10	*Launaea nudicaulis (Ln)*	15	16
11	*Euphorbia heterophylla (Eh)*	12-13	12-13
12	*Sphagneticola trilobata (Sp)*	20-21	20-21
13	*Ficus spp. (Fs)*	20-21	20-21

Table 5: Regeneration time data for all DNRR plant species. WT- With touch, WOT- Without touch, and DPC- Days Post Cut. The plants highlighted in light blue color are touch-independent plants, whereas those highlighted in light orange are touch-dependent plants. Each experiment was performed with one biological replicate.

Furthermore, the families *Asteraceae, Balsaminaceae, Lamiaceae, Scrophulariaceae*, and *Acanthaceae* are closely related. The time required for a plant to display its very first root regeneration (i.e., Regeneration time) was calculated. This regeneration time is different for different plant species (Table 5). Some of them exhibit the initial DNRR response as early as 6 DPC (days post-cut of the leaf), whilst others need 20 to 21 DPC. This data demonstrates the variation in the regeneration period of several plant species.

3.1.5 Requirement of physical contact at an early time point is necessary to show the DNRR response.

Physical contact (solid or liquid) at the cut end is crucial for determining the DNRR in touch-dependent plants. The question now is how long this contact at the cut end is necessary to show the DNRR. To check this, an experimental assay was designed where all the WOT samples were given touch to the growth medium at different time points (12H, 24H, 48H, 72H, 96H). For example, the 12H sample means that the sample retained 12H in WOT before switching to WT (Figure 14). Likewise, for the 24H, 48H, and so on. The touch-dependent plant *Centratherum punctatum (Cp)* was chosen for this study. All of the samples at different time periods (12Hrs, 24Hrs, 48Hrs, and 72Hrs) exhibit rooting phenotype. But, the sample at 96 Hrs didn't show

prominent rootings (Figure 14). This means that in plant *Cp* if the cut end is touched after 96 hours, the detached leaf will not be able to establish de novo roots. This 96

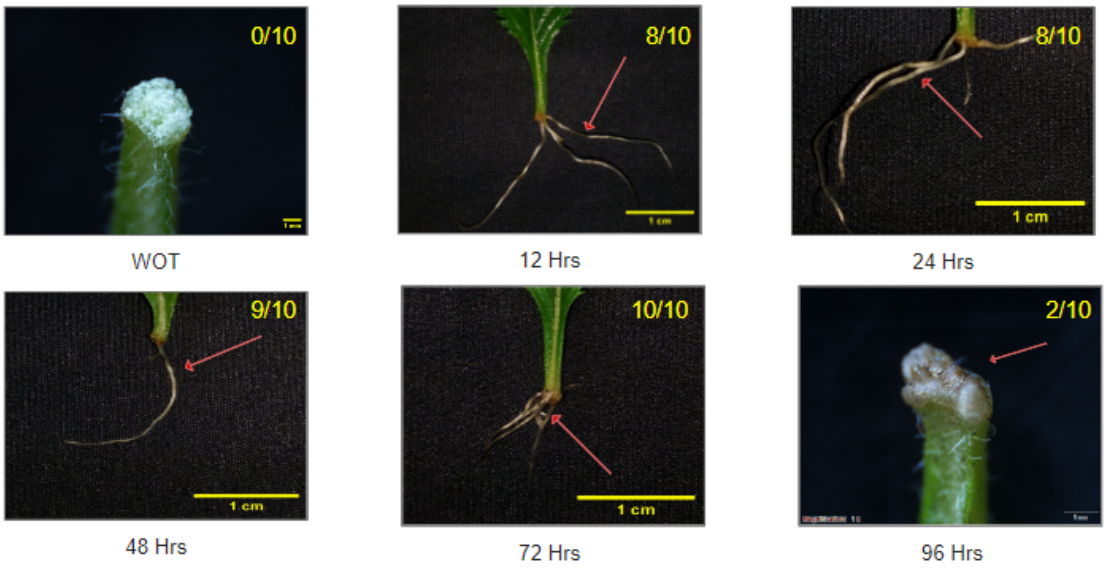


Figure 14: 96 Hrs time window for the touch-dependent plant *Centratherum punctatum (Cp).* The Without Touch/ Control (WOT) sample, 12Hrs WOT, 24Hrs WOT, 48Hrs WOT, 72Hrs WOT, and 96Hrs WOT sample. The regeneration frequency (i.e., Number of samples showing response/Total number of samples) is shown for the DNRR response. Samples were scored on day 14 post-cut.

time frame is required, after which the detached leaf fails to regenerate, demonstrating the relevance of the dynamic nature of physical touch at the cut end. This time frame may be different for each plant.

3.1.6 Mechanical injury plays an important role in the DNRR response.

When the leaf is disconnected from the plant, it is a type of primary mechanical injury. However, it is interesting to ask what will happen if an additional injury is given (i.e., secondary injury) to the leaf. To study the effect of the secondary injury, the incision is given at the junction of the leaf blade and the petiole at the same time of leaf cutting.

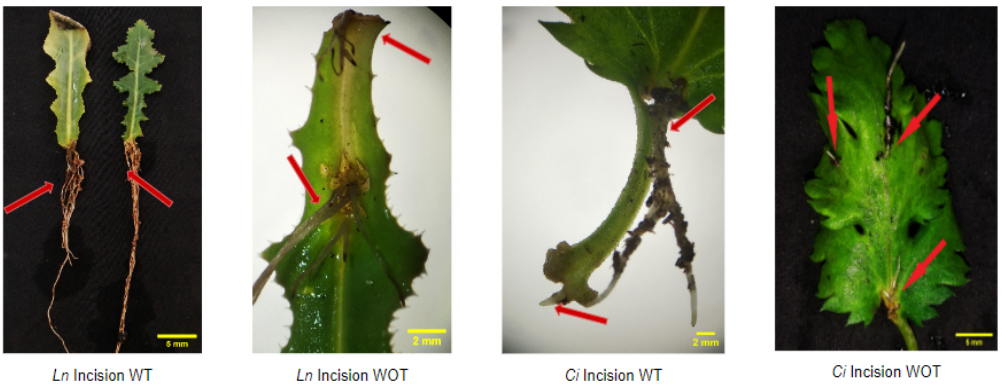


Figure 15: Secondary injury in *Launaea nudicaulis (Ln)* and *Chrysanthemum indicum (Ci).* Incision WT- secondary injury in the form of incision is given in With Touch sample. Incision WOT- secondary injury in the form of incision is given in the Without Touch sample.

In the plant *Launaea nudicaulis (Ln),* when the secondary injury is given in the WT sample, it produces multiple roots (Figure 15), in contrast to the primary injury, where it shows only a single root. In the WOT sample, however, *Ln* produces roots from the cut end as well as the petiole-leaf blade junction (Figure 15). A similar response is shown by the plant *Chrysanthemum indicum (Ci)*. Interestingly, incision WOT samples in *Ci* showed *de novo* roots from the mid-vein and lateral veins (Figure 15).

Another experiment was carried out to investigate the effect of secondary injury on the DNRR response. In the With Touch group, the leaf petiole was split into two halves and scored for regeneration response. Some plants had roots on only one side of the divided petiole (*Iw* and *Ci*). Others demonstrated roots from both split sides. (*Ps, Po*, and *Cp*). While some of them (*Ba* and *Pc*) did not show any roots at all (Figure 16).

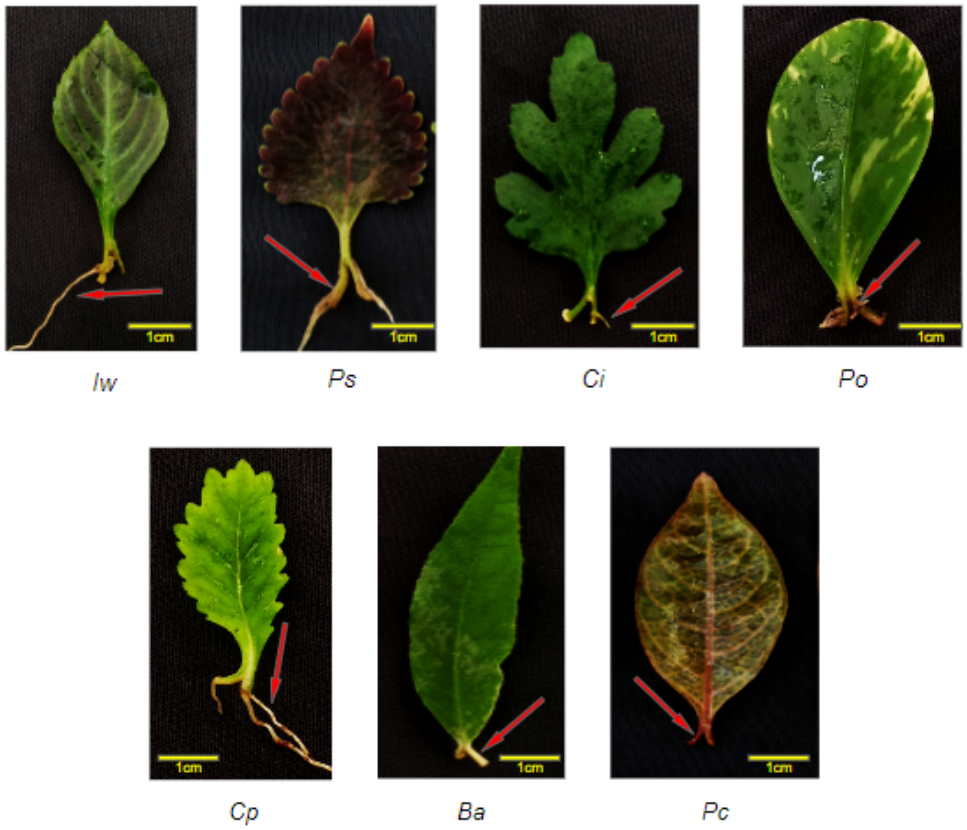


Figure 16: Petiole split experiment in different plant species. *Iw- Impatiens walleriana, Ps- Plectranthus scutellarioides, Ci- Chrysanthemum indicum, Po- Peperomia obtusifolia, Cp- Centratherum punctatum, Ba- Buddleja asiatica, Pc- Pseuderanthemum carruthersii.* Each experiment was performed with a single biological replicate, and each replicate contains five leaves.

A similar petiole split experiment was also carried out on the model plant *Arabidopsis thaliana* (*Col-Ikram*, WT). 33% of the total number of samples had roots from one split side of the petiole. (Figure 17). The remaining samples didn't show roots.

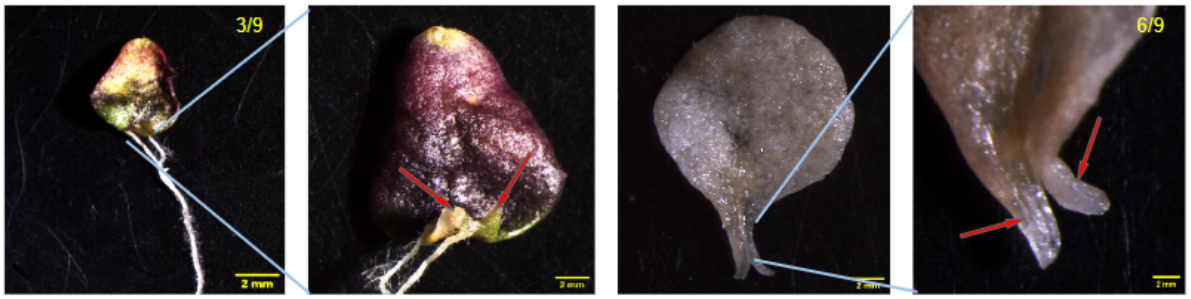


Figure 17: Petiole split experiment in *Arabidopsis thaliana (At) Col-Ikram*. A) At WT sample showing DNRR response from one side of the petiole. A') Magnified image of the DNRR sample. B) At WT sample showing no DNRR. B') Magnified image of the no DNRR sample. Growth medium- ½ MS agar medium. The numbers show the regeneration frequency (i.e., Number of samples showing response/Total number of samples).

3.1.7 Age-dependent distinctive fate switch in DNRR assay in *Arabidopsis thaliana*

Previous work has shown that the regeneration ability of the *Arabidopsis* younger leaf is higher, and it declines as the leaf ages (Chen *et al.*, 2014). However, the regeneration ability of very old leaves in the case of DNRR is not well studied. Therefore, a DNRR assay (similar to younger leaves) was performed for the 5 to 6-week-old plant leaves.

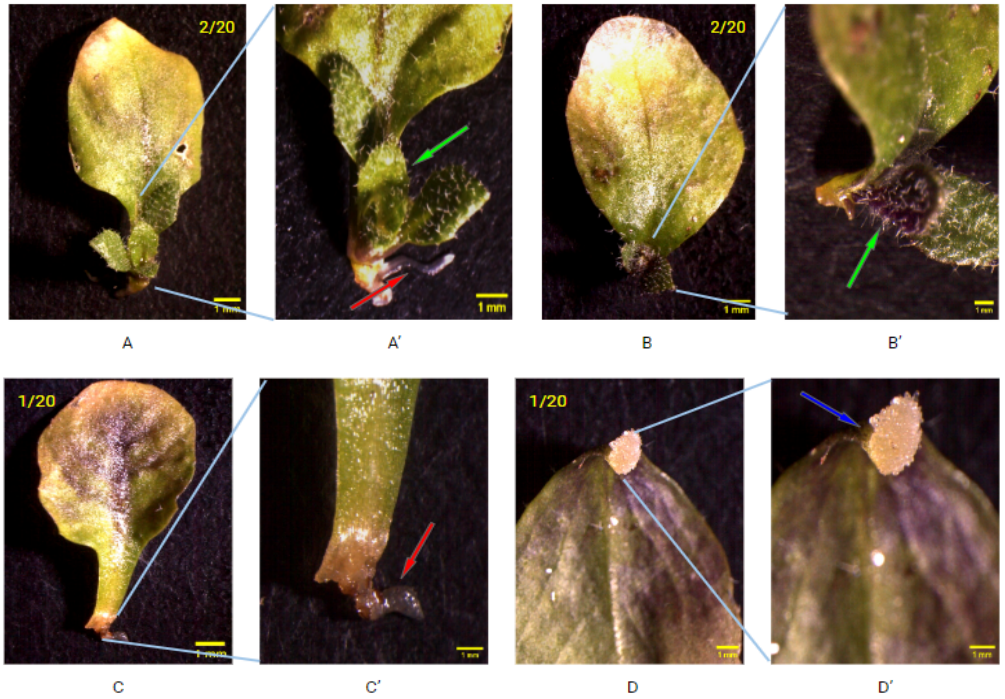


Figure 18: DNRR assay in the mature *Arabidopsis thaliana (At)* leaves. All the leaf samples were kept in the With Touch group. A, A') Detached leaf showing *de novo* shoot and *de novo* root. B, B') Detached leaf showing only *de novo* shoot and no root. C, C') Detached leaf showing *de novo* root. D, D') Detached leaf showing wound healing in the form of callus formation. All the leaves were taken from the 5-6 week-old *Arabidopsis Col-Ikram* plant. Red arrow- *De novo* root, Green arrow- *De novo* shoot, Blue arrow- Callus. The numbers show the regeneration frequency (i.e., Number of samples showing response/Total number of samples).

Out of 20 samples in the With Touch group, 2 samples showed the *de novo* shoot and *de novo* root organogenesis (Figure 18A). 2 samples showed *de novo* shoot only but no *de novo* root (Figure 18B). Here, the *de novo* shoot means there is the formation of new shoots from the cut end of the leaf. In addition to this, one sample showed typical *de novo* root response only (Figure 18C), and even one sample showed callus formation (Figure 18D). All the above regeneration responses are very different from earlier reported responses. It is quite impressive to see the regeneration response that older leaves show but not the younger ones.

Section 3.2 *PLETHORA (PLTs)* homologous genes in different plant species

3.2.1 Degenerate primers were designed to amplify the *PLETHORA (PLT)* genes

Designing primers against the target gene was the main challenge because the genome of the plants considered for the study was not available. So, the problem here is to amplify the gene of interest for which the sequence is unavailable. This problem can be approached by the method of designing degenerate primers.

A primer is called degenerate if various possible bases exist at one or more positions (Kwok *et al.*, 1994; Linhart and Shamir, 2007). The aim here is to design primers that will match the majority of the similar target sequences (for example, *PLT7* homologs), including the unknown sequences. If a primer must bind a large number of input sequences, it should be degenerate. However, if the degeneracy is severe, it is possible that the primer will result in non-specific amplification. That is also why degeneracy should be limited. This is known as a Degenerate Primer Designing (DPD) problem in primer design (Linhart and Shamir, 2007). As a result, there is always a middle ground between degeneracy and coverage. (i.e., the number of matched input sequences). The workflow of the degenerate primer designing for the *PLT7* gene is described below (Figure 19).

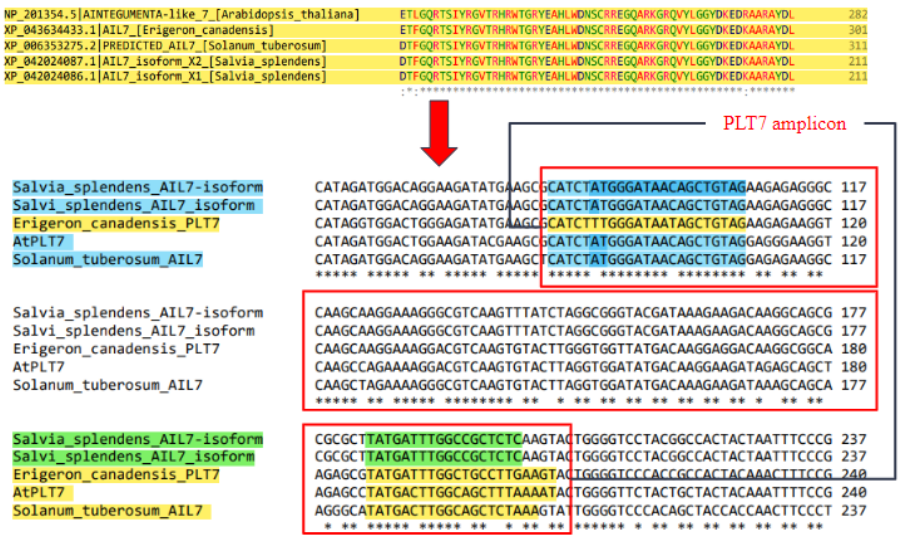


Figure 19: Workflow of the degenerate primer designed to amplify *PLETHORA7* (*PLT7*) homologous genes. First, the available PLT7 protein sequences were collected from the NCBI database. The most conserved amino acid region was identified by doing the multiple sequence alignment (MSA) of all the compiled sequences (highlighted in yellow). Then, this conserved a.a. region was reverse translated into the nucleotide sequence with the help of the available *PLT7* coding sequence (CDS) sequence of the corresponding protein sequence. Finally, the nucleotide sequence's most conserved region was found, and primers were designed to amplify *PLT7* homologous genes. (highlighted in red box). (See Methods sections 2.5)

3.2.2 *PLT7* homologous genes amplified in the different plant species using degenerate primers.

The gradient polymerase chain reaction (PCR) was used to assess the efficiency of degenerate primers in annealing and amplifying the *PLETHORA 7 (PLT7)* gene. To do the PCR, RNA was extracted from the plant's leaf petiole and converted into cDNA. This cDNA was used as a template in subsequent PCRs. All possible combinations of degenerate primers were tested in order to amplify the gene of interest. *PLT7* degenerate primers amplified the gene in *Centratherum punctatum (Cp)* (cDNA), *Impatiens walleriana (Iw)* (cDNA), *Mentha rotundifolia (Mr)* (cDNA), *Buddleja asiatica (Ba)* (cDNA), *Chrysanthemum indicum (Ci)* (cDNA), and *Sonchus (So)* (cDNA) (Figure 20).

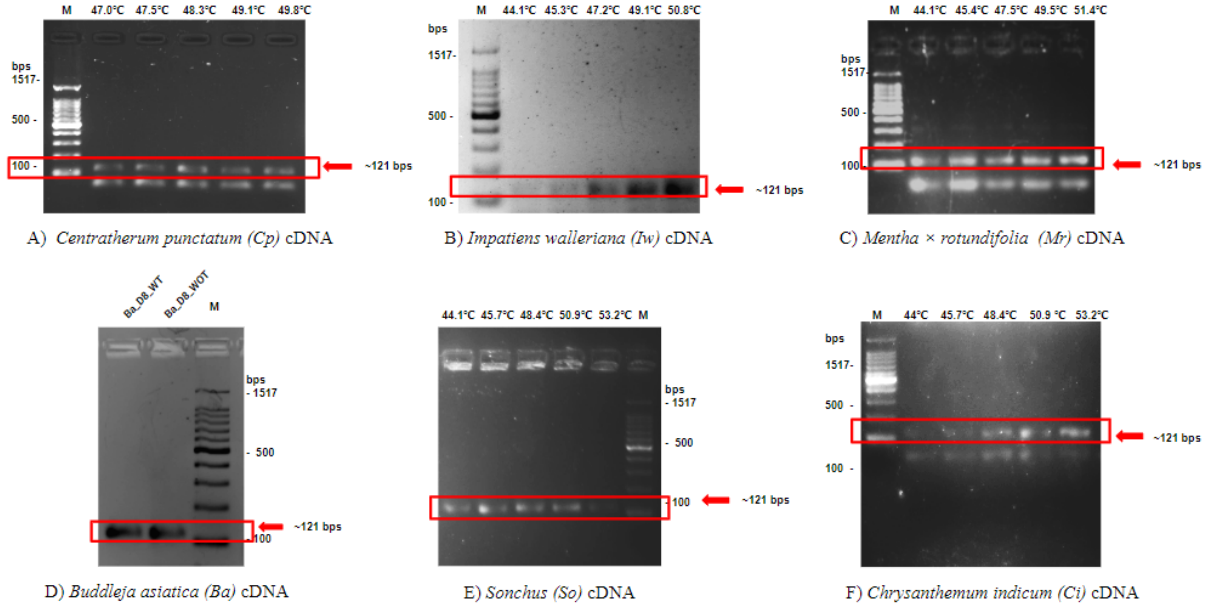


Figure 20: Amplification (~121 bps) from different plants using *PLT7* degenerate primers. A) *Centratherum punctatum (Cp)* cDNA (Primers set- Ec_PLT7FP, Ec_PLT7RP); B) *Impatiens walleriana (Iw)* cDNA (Primers set- Ec_PLT7FP, Ec_PLT7RP); C) *Mentha × rotundifolia (Mr)* cDNA (Primers set- Ec_PLT7FP, Ec_PLT7RP); D) *Buddleja asiatica (Ba)* cDNA (Primers set- ESt_PLT7FP, ESt_PLT7RP), E) *Sonchus (So)* cDNA (Primers set- Comm_PLT7FP, Comm_PLT7RP); F) *Chrysanthemum indicum (Ci)* cDNA (Primers set- Ec_PLT7FP, Ec_PLT7RP). The PCR products were separated on 1/2% agarose gels.

3.2.3 Sequencing results confirm the presence of *PLETHORA 7 (PLT7)* homologous genes in different plants.

The next objective was to sequence the amplicon of the previous PCR product to confirm the sequence homology with the *Arabidopsis PLETHORA 7* gene. The amplicon was sequenced and then aligned with the *Arabidopsis* CDS database using

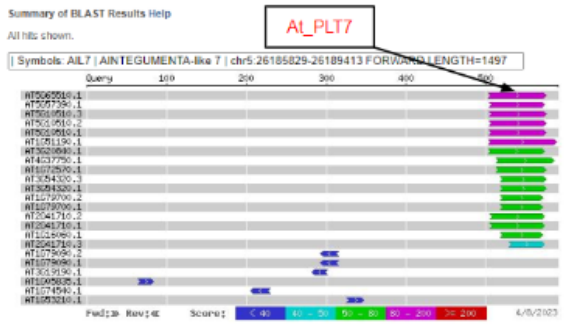
the TAIR BLAST Tool (https://www.arabidopsis.org/). The database chosen for the alignment was TAIR10 CDS (-introns, -UTRs) (DNA).

For the plant species *Centratherum punctatum (Cp)* (Figure 21A), *Buddleja asiatica (Ba)* (Figure 21B), *Sonchus (So)* (Figure 21C), *Impatiens walleriana (Iw)* (Figure 21 D), and *Mint (Mr)* (Figure 21E) the query sequences hit the *Arabidopsis thaliana (At) PLT7 (AIL7)* (*Cp* score= 95.1 bits, *Ba* score= 143 bits, *So* score= 134 bits, *Iw* score= 81.5 bits, *Mr* score= 45.5 bits) (bit score describes the overall quality of an alignment).

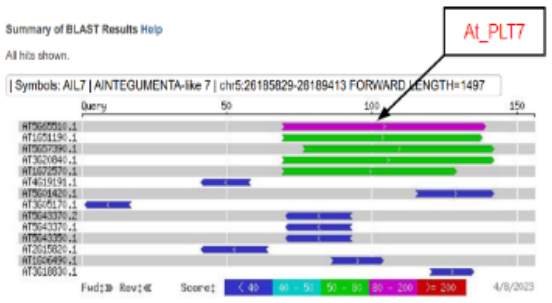
In *Cp*, the query sequence also hits *PLT2* (score= 80.6 bits), *PLT3* (score= 73.4 bits), *PLT1* (score= 65.3 bits), and *PLT5* (score= 44.6 bits), but with a lower score than *PLT7* (score= 95.1 bits). In *Ba*, the query hits *PLT3* (score= 87.8 bits), *PLT5* (score= 86.9 bits), *PLT2* (score= 73.4 bits), and *PLT1* (score= 67.1 bits) with a lower score than *PLT7* (score= 143 bits). In *So*, the query finds *PLT5* (score= 93.3 bits), *PLT3* and *PLT2* (score= 83.3 bits), and *PLT1* (score= 72.5 bits). *PLT2* (score= 61.7 bits), *PLT5* (score= 51.8 bits), and *PLT1* (score= 50.9 bits) all had significant scores for plant *Iw*. Finally, for *Mr*, the sequence hits *PLT1* (score= 43.7 bits), *PLT3*, and *PLT5* (score= 41.0 bits).

A) *Centratherum punctatum (Cp)*

B) *Buddleja asiatica (Ba)*



C) *Sonchus (So)*



D) *Impatiens walleriana (Iw)*

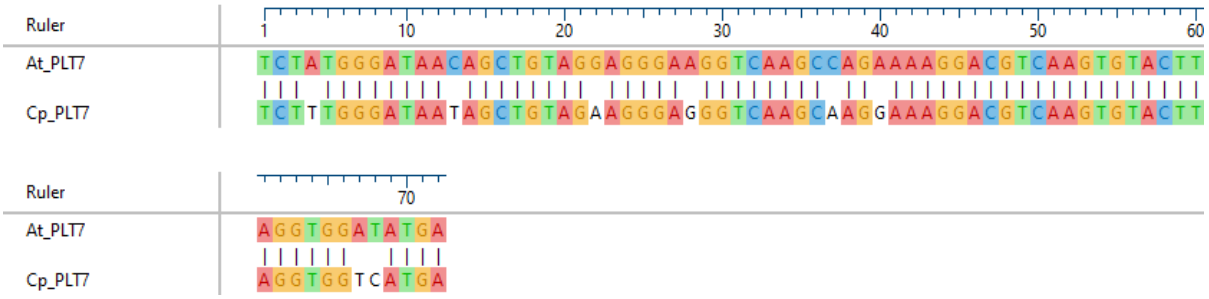
E) *Mentha × rotundifolia (Mr)*

Figure 21: TAIR blast tool sequence alignment results for A) *Centratherum punctatum (Cp),* B) *Buddleja asiatica (Ba),* C) *Sonchus (So),* D) *Impatiens walleriana (Iw)*, E) *Mentha × rotundifolia (Mr).* Database- TAIR10 CDS (-introns, -UTRs) (DNA).

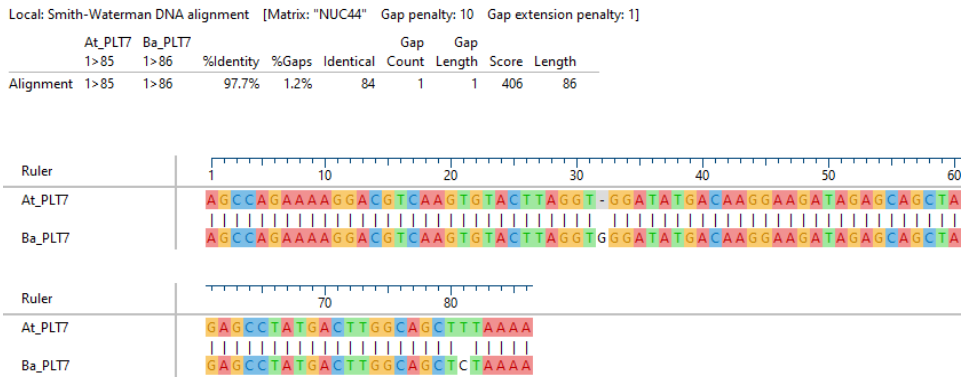
Following that, as the query sequences were amplified from the cDNA, they were pairwise aligned (PSA) with the *Arabidopsis thaliana (At) PLT7* CDS sequence. *PLTs* are members of the AP2/ERF transcription factor family, which includes the two AP2/ERF domains (AP2/ERF1 and AP2/ERF2). Interestingly, all of the query sequences exhibited close alignment with the PLT7 CDS AP2/ERF1 domain, which is shared by all *PLTs* (Figure 22). Multiple sequence alignment (MSA) of all *PLT7* homologous sequences with *Arabidopsis thaliana (A*t) *PLT7* CDS AP2/ERF1 domain was performed to confirm this. The results revealed a conserved domain in all PLT7 homologous sequences (Figure 23A). A tree was constructed using these aligned sequences (Figure 23B). *Mr* and *Ba* are plant sequences that are closely related to *Arabidopsis*. While *Iw* and *Cp* are closely related. *So*, on the other hand, has a very distant relationship with all other plant species (Figure 23B).

Local: Smith-Waterman DNA alignment [Matrix: "NUC44" Gap penalty: 10 Gap extension penalty: 1]

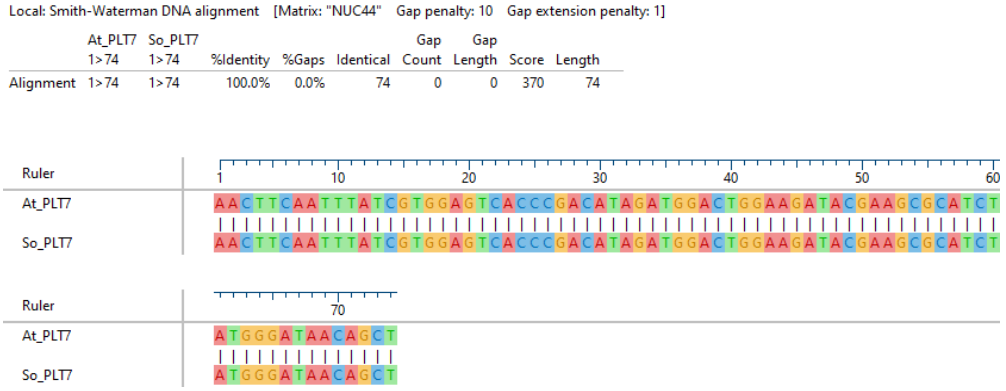
	At_PLT7 1>72	Cp_PLT7 1>72	%Identity	%Gaps	Identical	Gap Count	Gap Length	Score	Length
Alignment	1>72	1>72	88.9%	0.0%	64	0	0	288	72



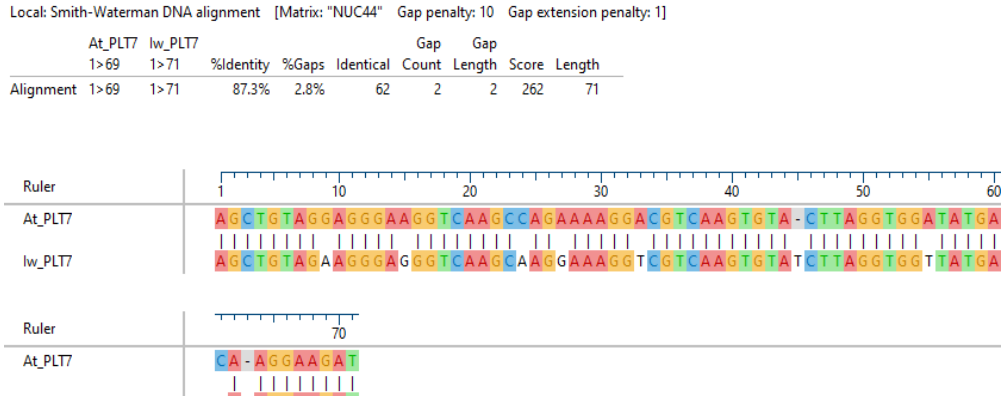
A) *Centratherum punctatum (Cp)*



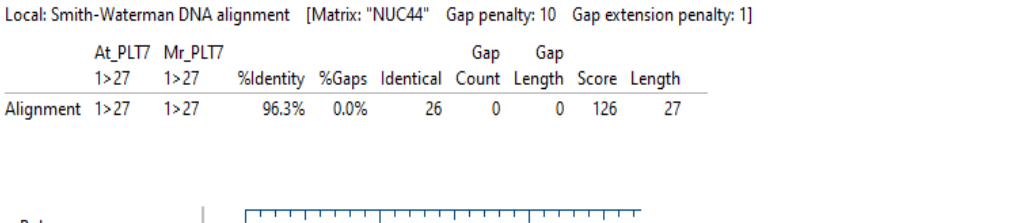
B) Buddleja asiatica (Ba)



C) *Sonchus (So)*

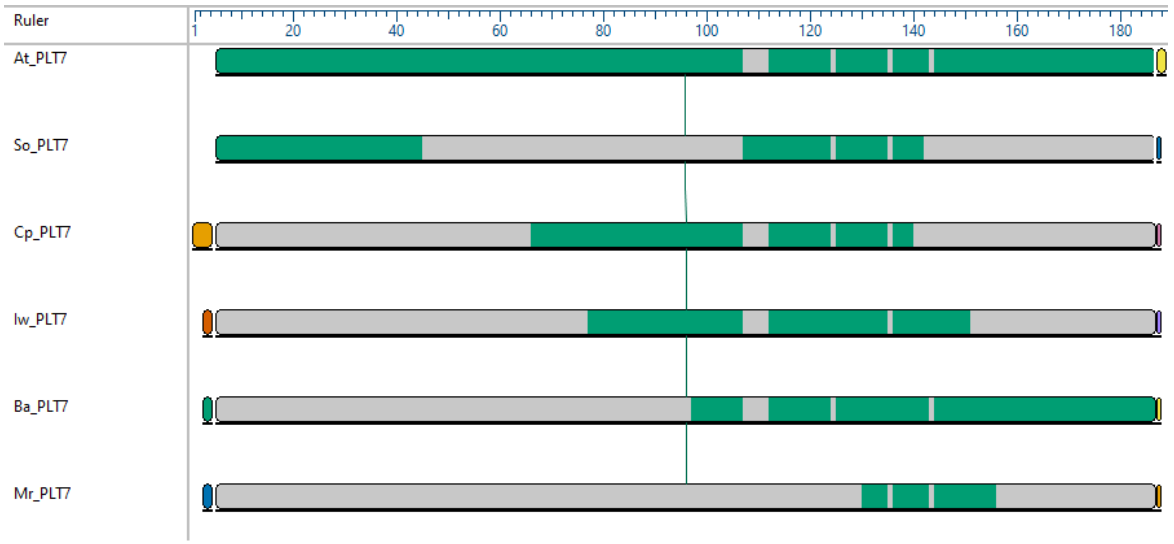


D) Impatiens walleriana (Iw)

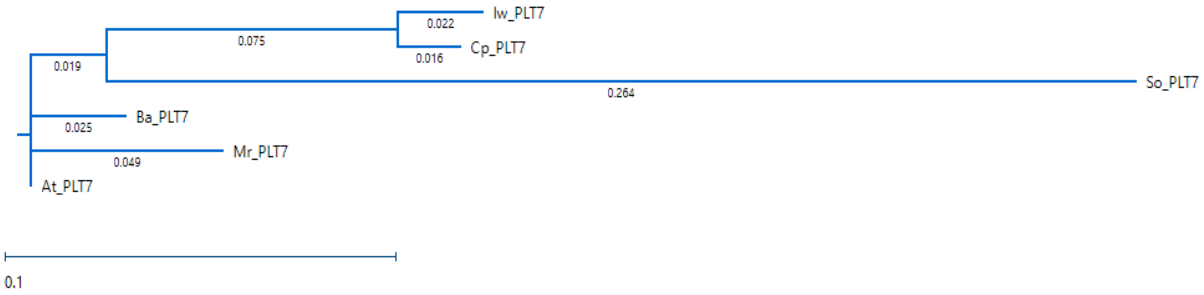


E) Mentha × rotundifolia (Mr)

Figure 22: Pairwise sequence alignment (PSA) of the different plant's *PLT7* homolog with the *Arabidopsis thaliana (At) PLT7* CDS sequence. A) *Centratherum punctatum (Cp),* B) *Buddleja asiatica (Ba),* C) *Sonchus (So),* D) *Impatiens walleriana (Iw)*, E) *Mentha × rotundifolia (Mr).* Sequence algorithm used: Local Smith-Waterman alignment.



A) Multiple sequence alignment (MSA) of all *PLT7* homologs



B) Tree showing a relationship between *PLT7* homologs

Figure 23: A) Schematic of Multiple sequence alignment (MSA) of all the plant species PLT7 homologs with *Arabidopsis thaliana (At) PLT7* AP2/ERF1 domain CDS sequence. The green color shows the conserved region of the alignment with the *Arabidopsis* PLT7 sequence. Sequence algorithm used: Mauve. B) Dendrogram showing a relation between the different plant species. The tree was constructed using the maximum likelihood method.

3.2.4 The sequenced *PLT* homologous gene expression pattern correlates with DNRR response.

Now, after the *PLT7* amplicon of the given plant species is sequenced, it is important to look into the involvement of the amplified homologous gene in *de novo* root organogenesis. To confirm this, semi-quantitative PCR of amplified gene products was performed to check the relative gene expression pattern in the given plant species.

As shown above, the DNRR timing of response of each plant varies (Table 5). For example, in the With touch (WT) samples, the touch-independent plant *Impatiens walleriana (Iw)* generates the first root (i.e., DNRR) 5 to 6 DPC (days post-cut). However, in Without Touch (WOT) samples, it takes 9 to 11 DPC to exhibit the regeneration response (i.e., DNRR). Similarly, on the 7th DPC, a touch-dependent plant, *Centratherum punctatum (Cp)*, exhibits first root (in WT) and callus (in WOT) responses. As a result, it is obvious that the regenerating time varies between various plant species.

To determine the role of the amplified and sequenced homologous *PLETHORA* genes, semi-quantitative PCR was done at various time periods, such as Day 0 and Day when the sample shows the regeneration response in WT and WOT (i.e., either DNRR or callus). The change in the relative gene expression of the *PLT7* homologous gene will reveal any correlation of amplified products in DNRR.

In touch-dependent plants, *Centratherum punctatum (Cp), Buddleja asiatica (Ba), and Mentha rotundifolia (Mr),* there is a correlation between gene expression at 0 DPC and 6 or 7 or 8 DPC for *Mr, Cp*, and *Ba*, respectively. (Figures 24A, 24C, 24D). In *Cp*, the 7 DPC With touch (WT) and 7 DPC Without touch (WOT) samples have

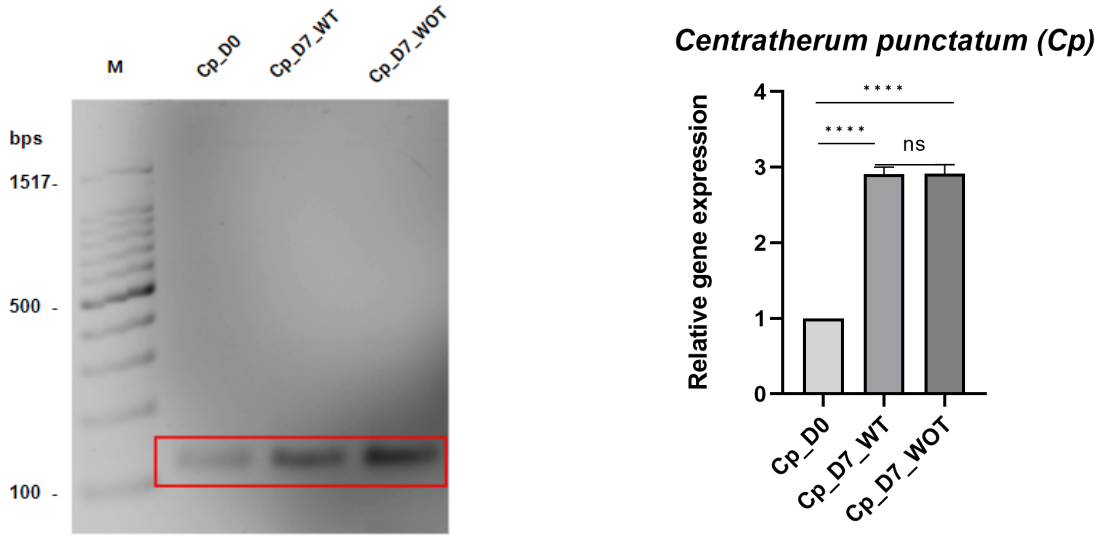


Figure 24 A): Semi-quantitative PCR for *Centratherum punctatum (Cp)*. LaneM- 100bp ladder, Lane2- D0: Hr post cut, Lane3- D7_WT: Day 7 post cut in With touch sample, Lane4- D7_WOT: Day 7 post cut in Without touch sample. Graph showing relative gene expression between different time points. Y-axis represents the grey value. (****P<0.0001, ns, P= 0.9741, Unpaired two-tailed t-test; n=4) n: no. of replicates, Error bar represents s.e.m.

higher relative gene expression than the 0 DPC samples. (Figure 24A). In *Ba*, the 8 DPC WT sample had higher expression than the 0 DPC sample. However, the 8 DPC WOT sample shows less expression than the WT sample (Figure 24C).

Interestingly, the model plant *Arabidopsis thaliana (At)* has a similar *PLT7* expression profile as in *Ba* (Figure 6).

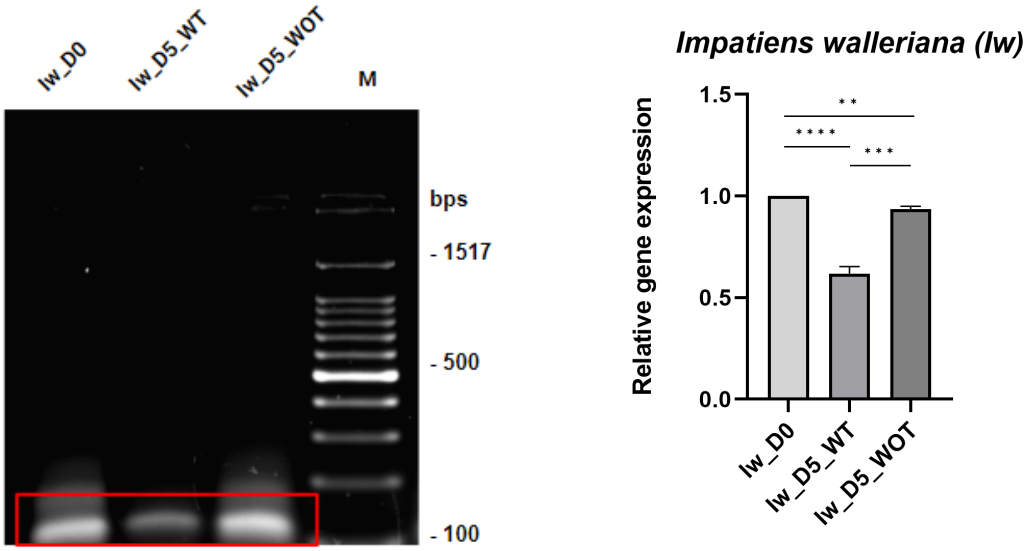


Figure 24 B): Semi-quantitative PCR for *Impatiens walleriana (Iw)*. LaneM- 100bp ladder, Lane1- D0: Hr post cut, Lane2- D5_WT: Day 5 post cut in With touch sample, Lane3- D5_WOT: Day 5 post cut in Without touch sample. Graph showing relative gene expression between different time points. Y-axis represents the grey value. (****P<0.0001, ***P=0.0001, **P=0.0041, Unpaired two-tailed t-test; n=4)

Impatiens walleriana (Iw), a touch-independent plant, has a significantly distinct expression profile than touch-dependent species (*Cp, Ba*, and *Mr*). Gene expression is higher in the 0 DPC and 5 DPC WOT samples than in the 5 DPC WT samples (Figure 24B).

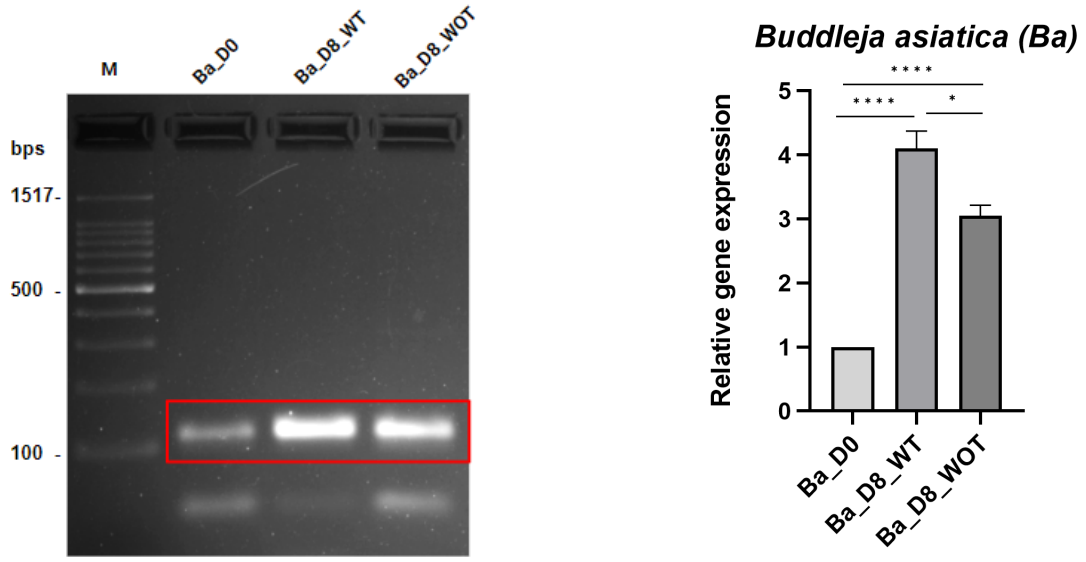


Figure 24 C): Semi-quantitative PCR for *Buddleja asiatica (Ba)*. LaneM- 100bp ladder, Lane2- D0: Hr post cut, Lane3- D8_WT: Day 8 post cut in With touch sample, Lane4- D8_WOT: Day 8 post cut in Without touch sample. Graph showing relative gene expression between different time points. Y-axis represents the grey value. (****P<0.0001, *P= 0.0165, Unpaired two-tailed t-test; n=4)

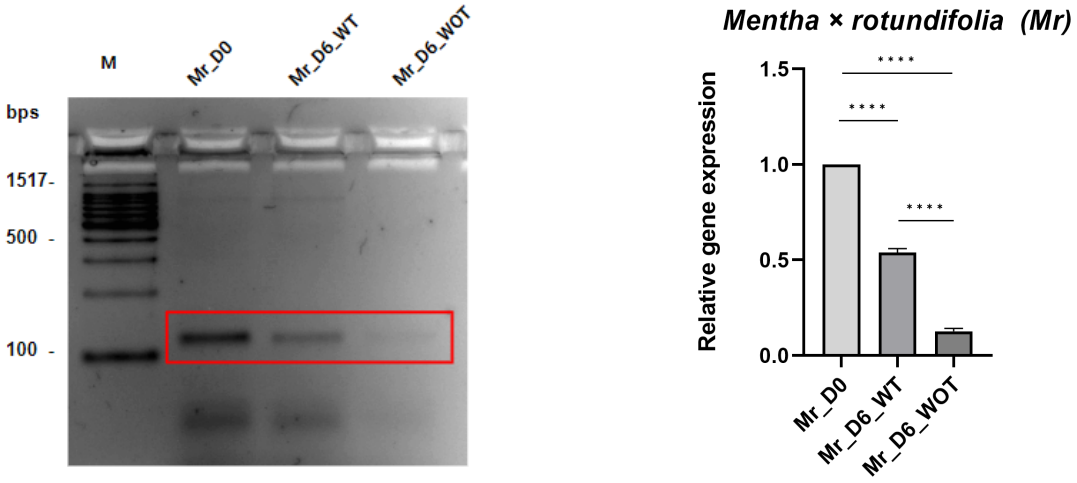


Figure 24 D): Semi-quantitative PCR for *Mentha × rotundifolia (Mr)*. LaneM- 100bp ladder, Lane2- D0: Hr post cut, Lane3- D6_WT: Day 6 post cut in With touch sample, Lane4- D6_WOT: Day 6 post cut in Without touch sample. Graph showing relative gene expression between different time points. Y-axis represents the grey value. (****P<0.0001, Unpaired two-tailed t-test; n=4)

Section 3.3 Polyclonal antibody production against PLT7 protein

3.3.1 Why antibodies against specifically PLETHORA 7 protein?

PLT proteins possess two AP2/ERF domains (AP2/ERF1 and AP2/ERF2) that are conserved among PLT proteins (Figure 25). PLT proteins are essential for *Arabidopsis thaliana*'s DNRR response. (Shanmukhan et al., 2021). Furthermore, PLT7 itself is sufficient for displaying the DNRR response among other PLTs (PLT3, PLT5, and PLT7) (Figure 7). Assuming PLT7 is a core TF, it should be conserved across plant species. It is more likely to find PLT7 homologous proteins in these plant species after detecting *PLT7* homologous genes in these plant species (See Section 3.2.2). This can be accomplished by generating a polyclonal antibody against *Arabidopsis thaliana* full-length PLT7 protein (i.e., anti-PLT7_Full length antibody).

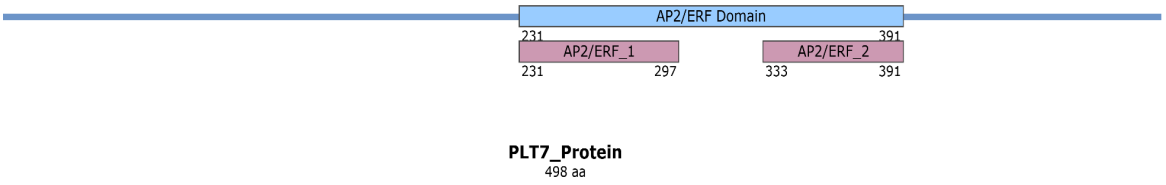


Figure 25: Schematic of PLT7 protein sequence containing the two AP2/ERF domains.

Since the PLT7 protein contains a conserved AP2/ERF domain, polyclonal antibodies against the conserved AP2/ERF domain (anti-PLT7_AP2/ERF domain antibody) can also be generated. As a result, an anti-PLT7_Full length antibody will recognize PLT7 homologous proteins, but an anti-PLT7_AP2/ERF domain antibody will recognize PLT-like homologous proteins that include the AP2/ERF domain.

3.3.2 Cloning full-length PLT7 and AP2/ERF domain PLT7 CDS in a vector

Two kinds of clones were generated to generate polyclonal antibodies against full-length PLT7 and AP2/ERF domain PLT7. The PLT7 recombinant protein was produced using an expression vector pET28a containing an N-terminal Histidine tag. The PLT7 gene expression vector with 6XHis-tag was constructed in accordance with the guidelines (See Section 2.8). PLT7 full-length CDS and PLT7 AP2/ERF domain CDS clones are designated pET28a.cPLT7 (Figure 26A) and pET28a.cPLT7.domain (Figure 26B), respectively. The plasmids pET28a.cPLT7 and pET28a.cPLT7.domain were then transformed into E. coli cells of the Rosetta strain.

Figure 26: A) Full-Length PLT7 protein clone (pET28a.cPLT7) and B) AP2/ERF PLT7 domain protein clone (pET28a.cPLT7.Domain)

3.3.3 Both recombinant proteins were synthesized with IPTG as an inducer.

To express full-length PLT7 and domain PLT7 protein peptide, different parameters for the expression were tried out. The full-length protein was induced at 37°C for 6 hours after IPTG induction at doses of 0.5mM, 0.6mM, and 0.7mM. (Figure 27A). In addition, the AP2/ERF domain protein was induced at 30°C for 6rhs after induction (Figure 27B).

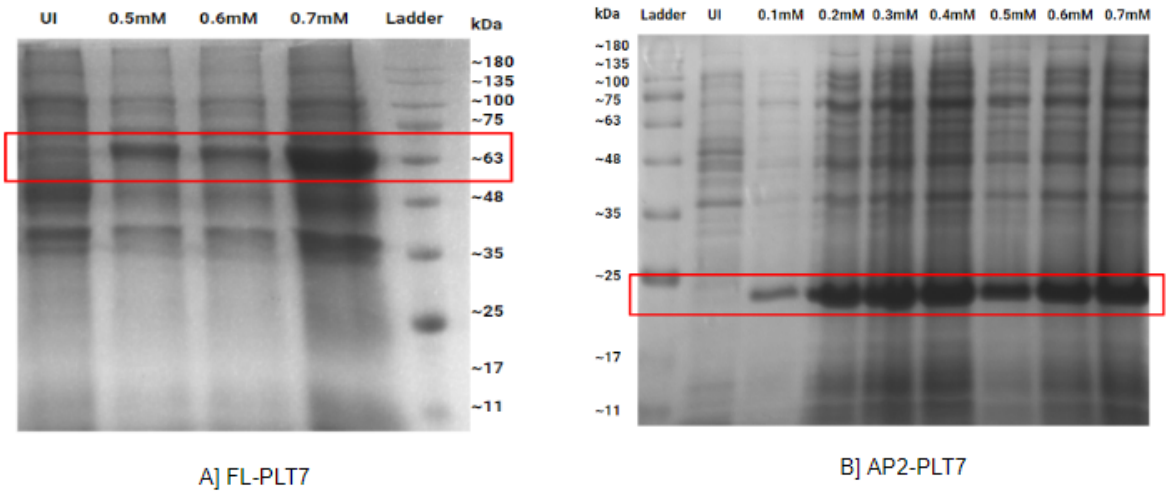
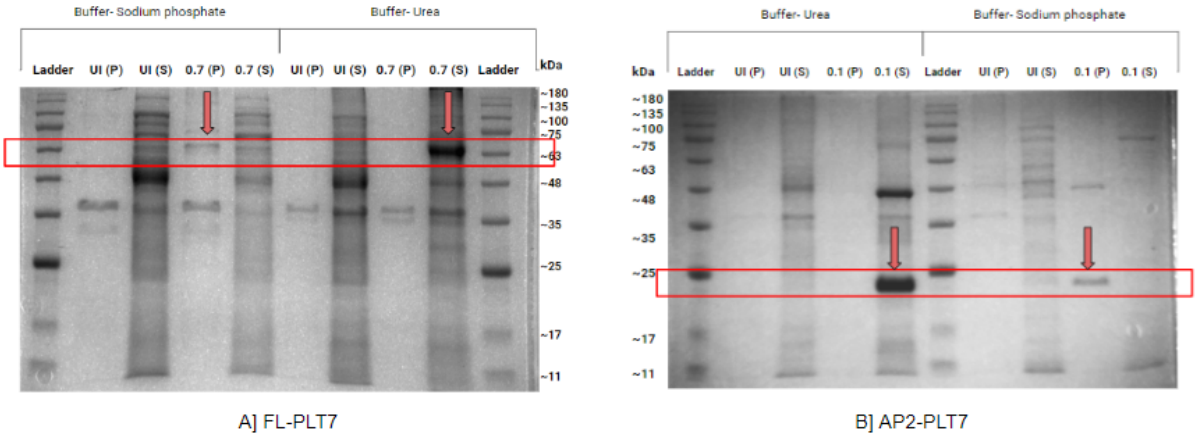


Figure 27: Induction for full-length PLT7 protein and AP2/ERF domain PLT7 protein peptide. A) FL-PLT7 (Molecular weight~55 kDa) IPTG concentration: 0.5 mM- 0.7mM, Tempe: 37℃, Time: 6 hrs post induction. Lane1- Uninduced (UI), Lane2- 0.5mM IPTG, Lane3- 0.6mM IPTG, Lane4- 0.7mM IPTG, and Lane5- Protein Ladder. B) AP2/ERF domain PLT7 (Molecular weight~18.5 kDa) IPTG concentration: 0.1mM- 0.7mM, Temperature: 30℃, Time: 6 hrs post induction. Lane1- Protein ladder, Lane2- Uninduced (UI), Lane3 to Lane9- 0.1mM- 0.7mM IPTG.

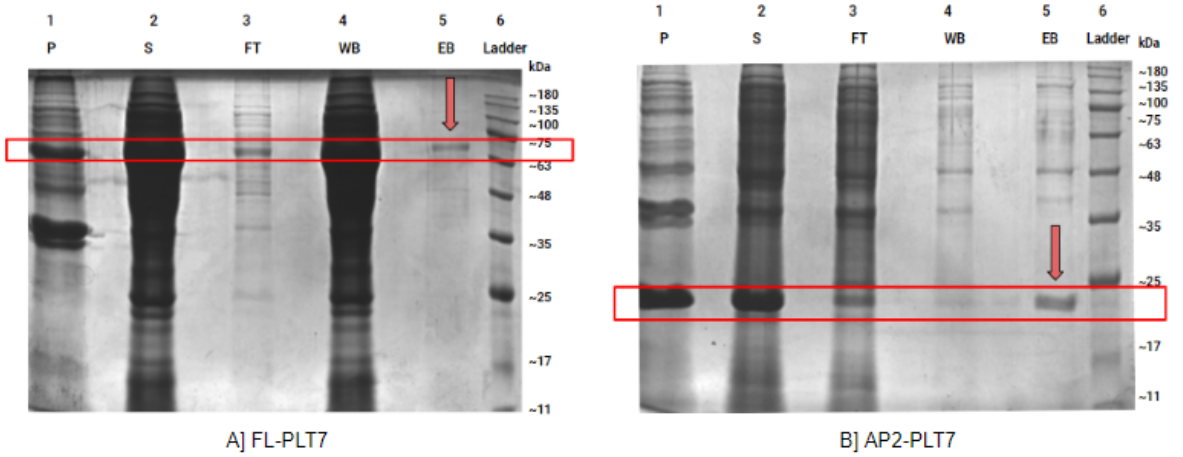
3.3.4 Proteins were purified using Ni-NTA affinity chromatography.

Antibodies can be raised against denatured proteins as well as native state proteins. First, both proteins were attempted to purify in the native state. As a result, several lysis buffers were utilized to lyse the cells. To get protein in its non-denatured state, sodium phosphate buffers were used. When the cells were lysed using sodium phosphate buffer, the entire expressed protein was found in the pellet rather than the supernatant (Figure 28).



A] FL-PLT7 B] AP2-PLT7

Figure 28: Cell lysis with Urea and sodium phosphate lysis buffer for full-length PLT7 protein and AP2/ERF domain-specific protein peptide. A) FL-PLT7, Sodium phosphate buffer [Lane1: Protein Ladder, Lane2: Uninduced (pellet), Lane3: Uninduced (supernatant), Lane4: Induced (pellet), Lane5: Induced (supernatant)]. Urea buffer [Lane6: Uninduced (pellet), Lane7: Uninduced (supernatant), Lane8: Induced (pellet), Lane9: Induced (supernatant), Lane10:Protein Ladder]; Cells were induced at 0.7mM of IPTG. B) AP2/ERF domain PLT7, Sodium phosphate buffer [Lane1: Protein Ladder, Lane2: Uninduced (pellet), Lane3: Uninduced (supernatant), Lane4: Induced (pellet), Lane5: Induced (supernatant)]. Urea buffer [Lane6: Uninduced (pellet), Lane7: Uninduced (supernatant), Lane8: Induced (pellet), Lane9: Induced (supernatant), Lane10:Protein Ladder]; Cells were induced at 0.1mM of IPTG. (Sonication parameters 1 sec on, 3 sec off, Amp- 60%, Time- 1-2 mins).



A] FL-PLT7 B] AP2-PLT7

Figure 29: Ni-NTA agarose beads purification with Urea Buffer. A) FL-PLT7, Lane1: Pellet (P), Lane2: Supernatant (S), Lane3: Flow through (FT). Lane 4: Wash buffer (WB), Lane 5: Elution buffer (EB); Lane 6: Ladder. B) AP2/ERF domain PLT7, Lane1: Pellet (P), Lane2: Supernatant (S), Lane3: Flow through (FT); Lane 4: Wash buffer (WB); Lane 5: Elution buffer (EB), Lane 6: Ladder. The eluted protein of interest is highlighted by a red arrow.

To get protein in its non-denatured state, urea buffers were used. When cells were lysed with urea buffer, all of the expressed protein was found in the soluble fraction (Figure 28). The denatured protein can be used as an antigen in the manufacture of polyclonal antibodies (Jenik and Irish, 2001). As a result, the full-length (FL-PLT7) and AP2/ERF (AP2-PLT7) domain proteins were denatured and purified in a lysis solution containing 8M urea (See methods section 2.9).

After standardizing the lysis buffer and induction settings for protein purification, bacterial cells were lysed with the following sonication parameters: 1 sec on, 3 sec off, Amplitude- 60%, Time-1/2 mins. FL-PLT7 and AP2-PLT7 induced protein came in the soluble fraction with 8M urea (Figure 29). Finally, both protein types were purified using Ni-NTA affinity chromatography. (See Section Methods section 2.9). Both 6XHis-tagged proteins were eluted with Imidazole, as shown in the SDS-PAGE analysis (Figure 29). Both proteins (FL-PLT7 and AP2-PLT7) were effectively purified; however, the eluted proteins were not pure because additional proteins were present in the eluted fractions. As a result, the proteins can be eluted in the Imidazole gradient and obtained in their pure form. This purified protein can then be utilized to produce polyclonal antibodies.

Chapter 4 Discussion

Mechanically induced regeneration can be seen by means of *de novo* root organogenesis (DNRR) from the cut end of a detached plant leaf (Mathew and Prasad, 2021). Plants are subjected to multiple types of injury and damage, whether natural or in vitro. Plants have evolved to survive these injuries and wounds by displaying various regeneration responses (e.g., shoot or root regeneration). In the mechanically injury-induced regeneration type, where a wounded/injured organ is still connected to the parent, the nutrient and water availability remains. However, in the case of DNRR from a detached organ, when the leaf explant is separated from the parent plant, that leaf must survive without any link to the parent plant (i.e., without nutrients and water availability from the parent). This is analogous to the natural scenario in which the leaf detaches from the plant and falls on the ground's surface, forming roots and shoots and eventually giving life to a new plant. Novel approaches for mimicking the natural DNRR response have been developed (Chen *et al.*, 2014; Shanmukhan *et al.*, 2021). We can use these methods to analyze the DNRR response and acquire an improved understanding of the mechanism underlying this type of regeneration.

This approach to studying DNRR is well established in the model plant *Arabidopsis thaliana*. As a result, the objective of this study was to detect similar DNRR responses among the plant species selected for the study. The results reveal that all of the plants responded positively to the DNRR test in two separate ways (Figures 10 and 11). One is comparable to *Arabidopsis* (touch-dependent), while another is not (touch-independent) (Figure 12). These findings complement previous findings (Mhimdi and Pérez-Pérez, 2020) that the ability to generate new adventitious roots (ARs) is conserved across these eudicot plants.

The unique feature of the DNRR in *Arabidopsis* is its touch-dependent nature (i.e., WT- Root, WOT- Callus). A detached leaf must make physical contact with a solid or liquid surface in order to regenerate the root (Shanmukhan *et al.*, 2021). There is a time frame for giving this touch to the cut end of the leaf, beyond which it will fail to establish the root (Figure 14). Surprisingly, some of the plant species tested for DNRR are touch-independent in nature (Table 3). (i.e., WT- Root, WOT- Root). Hence, those plants do not require a physical touch to form roots. From the

phylogenetic analysis of the given plants, it is clear that they belong to diverse families (Figure 13). All the above results suggest that the mechanically injury-induced DNRR regeneration response is a conserved mechanism as well as there also exists variation in regenerative response that could be arising because of the plants' diversity (Jayakumar and Nair, 2013; Subashree *et al.*, 2021). E.g., touch-independent plants might have evolved differently than touch-dependent plants throughout the course of evolution to show distinctive regenerative responses.

From the different experiments, such as giving secondary injury and splitting the petiole, it is certain that mechanical injury plays an important role in enhancing the regeneration response of the plant (Figure 15-17). It is clear from the experiments, such as giving secondary injury and splitting the petiole, that mechanical injury plays an important role in enhancing the plant's regeneration response. In contrast to prior work (Sun and Zhu, 2021), *Arabidopsis thaliana (At) de novo* shoot and root regeneration response from older leaves exhibited a completely novel regeneration phenotype (Figure 18). This gives assurance to the notion of an age-dependent distinctive fate switch in DNRR response.

The next question is, what is the origin or molecular foundation of this form of *de novo* organogenesis? Taking inspiration from the model plant *Arabidopsis*, I looked at the factors (*PLETHORA- PLTs*) crucial for DNRR in other plant species. With the use of degenerate primers, it is possible to amplify the *PLETHORA 7 (PLT7)* homologous genes in a few plant species. (*Iw, Cp, Mr, Ba, So*) (Figure 20). Furthermore, after sequencing the amplified gene, it is clear that *PLT or PLT7* homologous genes exist in these plant species, and the AP2/ERF1 domain is conserved in the given plant species as the query sequence hits other *PLTs* also (Figure 22).

After confirming the *PLT* homologous genes, it is necessary to show that they have any correlation in the DNRR response. The results of the semi-quantitative PCRs show a change in the relative gene expression of the *PLT* homologous gene in the given plants. There is either upregulation or downregulation of the *PLT7* homologous gene at different time points (i.e., 0 DPC, 5/6/8 DPC WT, and WOT). *Buddleja asiatica (Ba)*, a touch-dependent plant, has a *PLT7* gene expression profile similar to *Arabidopsis*. This may be linked to the touch-dependent character of both plants since the gene is expressed more in the WT response than in the WOT response. In *Centratherum punctatum (Cp),* WT and WOT exhibit higher expression than 0 DPC,

indicating that this *PLT7* homolog has some correlation in the DNRR response. In contrast to *Cp*, *the PLT7* expression of the WT and WOT in *Mentha rotundifolia (Mr)* is less than 0 DPC. It is probable that the *PLT7* gene expresses earlier rather than later stages. *Impatiens walleriana (Iw)* has a distinct profile from other plants, with WT expressing less than WOT. This might be due to the plant's lack of sensitivity to touch. The *PLT7* gene doesn't show increased expression in the WT response because the cut end is already in contact with the growth media, but there is no physical contact in the WOT response. Therefore, the *PLT* genes have to express more in order to compensate for the lack of touch. Now, do touch-independent plants really independent of touch? Because the results show that the average number of roots in the WT is more than WOT in touch-independent plants (Data not shown). This implies that even if the plant is touch-independent, the physical contact at the cut end enhances the DNRR response in WT. In summary, the variation seen at the phenotypic level may also exist at the genotypic level. Also, the tree constructed based on the *PLT7* sequences (Figure 23B) has close resemblance with the phylogenetic tree constructed at the family level (Figure 13). It supports the idea that different plants will respond differently in the context of DNRR. However, certain regeneration factors will be conserved.

Finally, similar to previous investigations (Kramer and Irish, 1999), a strategy of generating polyclonal antibodies against At-PLT7 was proposed to detect PLT7 protein in given plants. Clones were created using an expression vector with an N-terminal histidine tag for the production of full-length and AP2/ERF domain proteins, and both proteins were successfully produced in E. coli cells (Figure 28). The proteins were purified using affinity chromatography. In the future, after purifying the full-length and AP2/domain PLT7 proteins in pure form, the purified proteins will be used for polyclonal antibody production, and the antibody will be used to identify PLT or PLT7-like proteins.

"*PLETHORA* guided Universal regulatory module of regeneration?"

The transcription factors (TFs) *PLETHORAs (PLTs)* play an important part in several types of regeneration, including DNRR. *PLT7*, in particular, might be a master regulator of DNRR destiny since it is both adequate and essential for DNRR. This project's phenotypic, sequencing, and semi-quantitative data demonstrate the fact that these *PLT* factors are conserved across plant species and may function in the molecular network to produce responses like *de novo* root. If all of the above

information is right, there is a possibility that *PLETHORA* (particularly *PLT7*) directed universal regulatory module of regeneration is maintained across plant species.

Future aspects

A basic method for studying DNRR is extremely helpful in understanding how plant regeneration occurs. However, there are numerous unsolved questions: Why is it necessary for the cut portion of the leaf to make contact with its surroundings in order for new roots to form? At the cut end, what form of mechano-sensing occurs? What distinguishes the touch-dependent plant from the touch-independent plant? To find answers to these problems, greater research into the cellular and molecular origins of the DNRR in the model plant *Arabidopsis thaliana* is required. Furthermore, studying regeneration in a number of plant species allows us to understand regeneration from the perspective of evolution.

Limitations of the study

This study showed *PLT7* homologs in some but not all DNRR plant species, as other plants did not show any *PLT7* amplification. It could be because of primers that are degenerate which have both possibilities of binding to the target or not. It was also challenging to isolate RNA from the petiole sections of the plant leaf as some plants contain secondary metabolites. While screening the plant species, there are some other regenerative responses, such as callus formation from the cut end in With Touch or in the secondary injured leaves. Because this study focused on the DNRR, such responses were not investigated further.

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