Probing the role of reactive oxygen species (ROS) in plant regeneration

A Master's Thesis

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Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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From May 2023 to Mar 2024

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Certificate

This is to certify that this dissertation entitled '**Probing the role of Reactive Oxygen Species (ROS) in plant 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 **Amish Ashok Butle** at Indian Institute of Science Education and Research under the supervision of **Dr. Kalika Prasad**, Associate Professor, Department of Biology, during the academic year 2023-2024.

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This thesis is dedicated to Dr. B. R Ambedkar, Parents, Myself and lastly to you for reading this thesis.......thank you!

Declaration

I hereby declare that the matter embodied in the report entitled “**Probing the role of Reactive Oxygen Species (ROS) response in plant regeneration**” are the results of the work carried out by me at the Department of **BIOLOGY**, Indian Institute of Science Education & Research (IISER) Pune, under the supervision of **Dr. Kalika Prasad**, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

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Abstract

Plants have a remarkable capacity for wound-healing and regeneration, which can be investigated easily in *Arabidopsis thaliana*. Regenerative responses in Arabidopsis are broadly categorized as tissue culture-induced, or mechanical injury-induced regeneration. Regardless of the stimulus, regenerating tissue shows various stress responses before showing any visible changes in its morphology. Reactive oxygen species (ROS), accumulate as byproducts of aerobic metabolism, whose excess levels are harmful to plants, but they also serve as signalling molecules that are necessary for plants to initiate a repair and healing response. In order for organisms to survive and regenerate, ROS homeostasis must be tightly regulated. However, little is known about the in vivo spatiotemporal nature of ROS production and mitigation. In this study, we have used *A. thaliana* as a plant model system for studying the role of reactive oxygen species (ROS) in the plant regeneration.

In this project, the mutant lines of stress marker family called Rboh (Respiratory burst oxidase homolog) are used to study ROS in leaf vein regeneration assay and *de novo* root regeneration (DNRR) assay. Also, the ROS responsive fluorescent reporter was cloned, utilizing the promoter of a well-known ROS-responsive transcription factor ZAT12 to drive the expression of free mCherry (Red fluorescence protein), in order to understand the dynamics of ROS and it's biological role in plant regeneration.

None of the ROS mutants (*rbohD* and *rbohF*) were found to be impaired in leaf vein regeneration. Strikingly, besides D-loop which is seen in wild type, a straight path was also observed in some of the regenerated samples for both the mutants. H_2DCFDA fluorescent dye was used to monitor ROS dynamics post injury in these two mutants. ROS accumulation was observed in the cells lying in the immediate vicinity of the wound in wild type but not in *rbohD* and *rbohF* mutants. In case of DNRR, regeneration efficiency was severely compromised in these mutants. How ROS production signalling integrates with the auxin signalling and microtubules dynamics is still unclear. Generation of auxin and microtubule reporter lines in the *rbohD* and *rbohF* mutant background to study this integration is ongoing.

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Contributions

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

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Chapter 1 Introduction: Regeneration

1.1 Regeneration in Organisms

Irrespective of evolutionary origin, all living organisms discovered till now share an innate power which is nothing but the ability to grow and survive. But the growth and survival rate often get compromised which in most of the circumstances is due to the frequent injuries. Thus, to surpass such many more damaging effects, most of the living organisms have evolved a strategy called regeneration. Regeneration is the process of renewal, restoration, and growth that restores or renews injured or destroyed cells, tissues, organs, or even the entire body. Regeneration can be considered conserved but is very limited in higher organisms in the animal kingdom (Poss., 2010). The animal model organisms such as planaria, hydra, axolotl, tadpole, etc. can be used to study the regeneration and it can be noted that the regenerative property decreases with an increase in the level of complexity of the organism (Fig. 1).

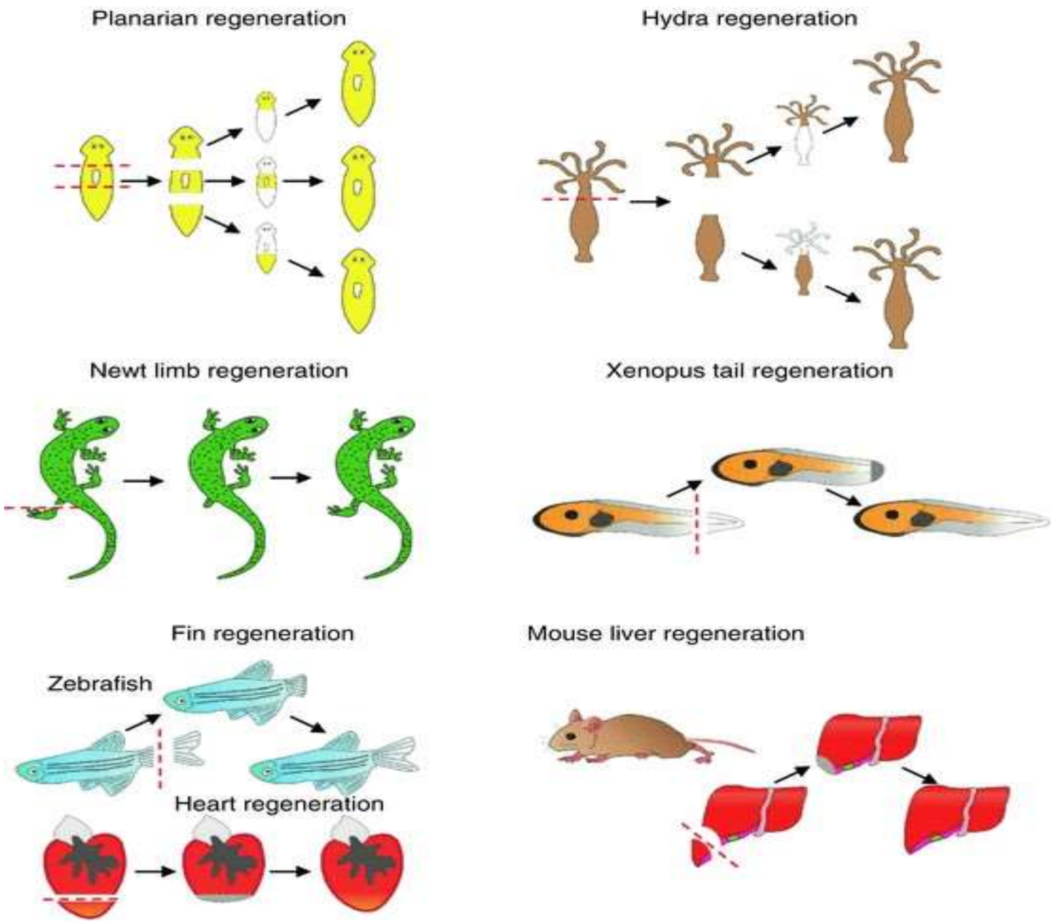


Fig. 1: Regeneration in Animal Kingdom. (Image Courtesy: Fu et al., 2018)

1.2 Regeneration in Plants

Plants have specific and robust regeneration responses that distinguish them from animals. After organ injury or loss, they show a variety of regeneration responses (Birnbaum and Alvarado, 2008), via various body components, such as leaves, stems, roots, hypocotyls, and so forth into new organs or change the identity of already existing organs. "The formation of shoots and/or roots from the damaged or lost part of the organ is the main characteristic of plant regeneration."(Ikeuchi and colleagues, 2019). In order to learn more about the elements that affect regeneration and how this information may be applied to the agricultural sectors, it is essential to comprehend the molecular and cellular foundation of regeneration.

In *A. thaliana*, Regenerative process can be divided into two categories.:

1) **Tissue culture-induced regeneration**: - This entails the *de novo* regeneration of an organ or fully developed plant from an explant. This phenomenon is referred to as *De novo* organogenesis. It can occur indirectly, in which an intermediary stage called callus is produced or directly, without the formation of callus. The former further can be divided into *de novo* shoot organogenesis and root organogenesis.
    a) *de novo* shoot regeneration- formation of new shoot from the explant
    b) *de novo* root regeneration- formation of new root from the explant

2) **Mechanical injury-induced regeneration** - During normal development, plants display a variety of mechanically driven regeneration responses in all parts of their bodies. For example, developing roots can be affected by biotic and abiotic stimuli, which often results in the loss of root tip or even individual root cells. (Durgaprasad *et al.*, 2019).
    a) First, when the organ is still attached to the main parent plant body.
    b) Second, when the organ is detached from the main parent plant body. Both aerial and underground organs can show regeneration.

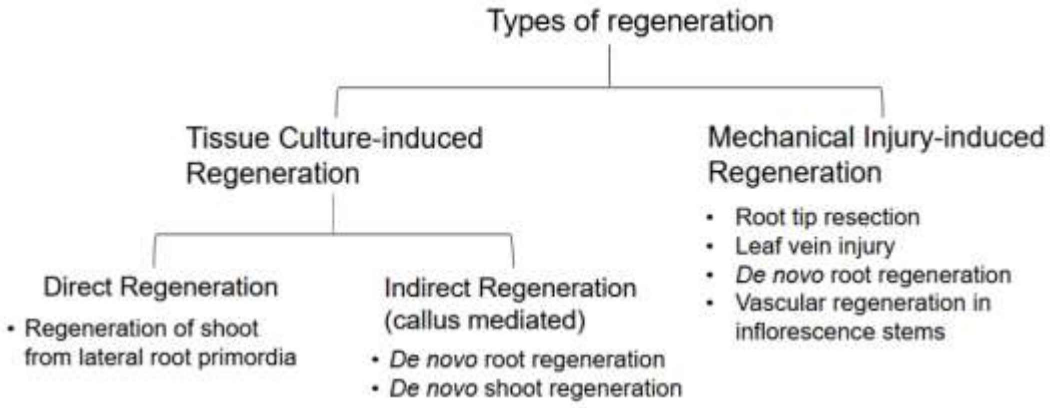


Figure 2. Types of regenerative responses in *Arabidopsis thaliana*. All the regenerative responses can be divided into tissue cultured induced regeneration and mechanical injury-based regeneration (MathewandPrasad,2021)

Now, Mechanical injury-based regeneration has been studied using the following regenerative processes (Figure 2) -

- *De novo* root regeneration
- Leaf vein injury
- Wound healing and vascular regeneration
- Root tip regeneration

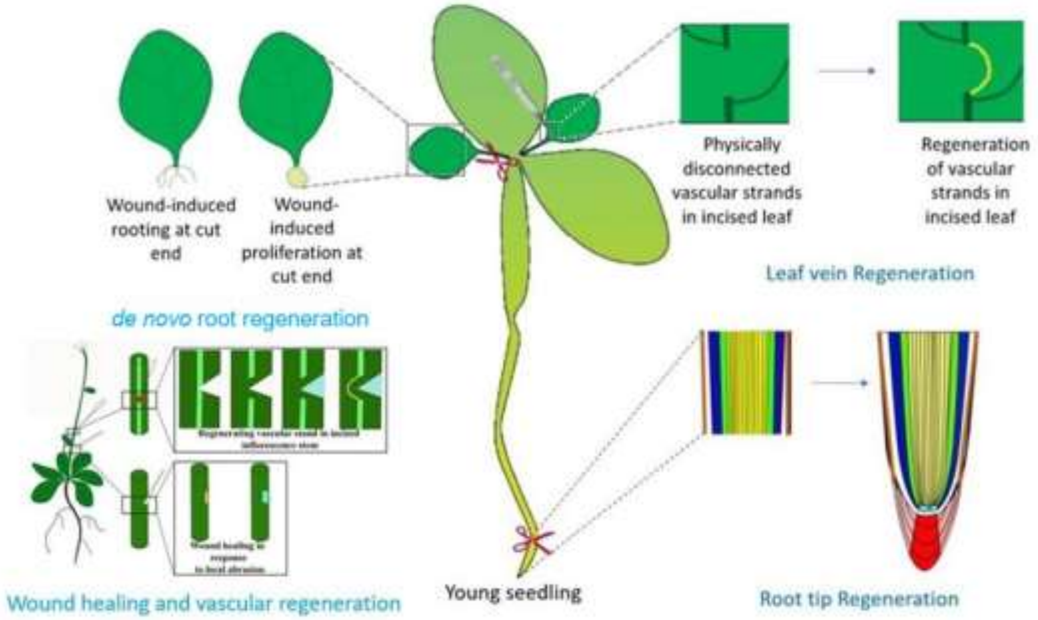


Figure 3. Schematic representation of different types of mechanical injury-based regenerative responses in Arabidopsis. **(A)** *De novo* root regeneration. **(B)** Leaf vein regeneration **(C)** Healing of wound and regeneration of vascular strands in the floral stems of *Arabidopsis thaliana*. Incision of the inflorescence stem interrupts the continuity of the vascular tissue and causes cell growth to fill the space left by the disconnected tissues. New vascular stands grow surrounding the injured location then restore vascular continuity. In contrast, only local cell growth is induced when the inflorescence stem's surface is scratched. **(D)** Regeneration at the root proximal tip and its cell. After the excision of the proximal region of root tip, non-injured endodermal/stelar cells at the cut end site multiply and temporarily activate embryonic guiding molecular passages pathways to replace the lost or damaged root proximal region (Mathew and Prasad, 2021).

1.3 *De novo* root regeneration (DNRR)

When the body part detaches from the main parent body plant, second form of injury i.e., mechanical injury-induced regeneration takes place. Research conducted on the plant model organism called *Arabidopsis thaliana* has demonstrated that *de novo* organogenesis, or the newly formed organ having a separate or unique character from the original tissue or organ, occurs when an organ or component is removed from the parent plant (Bustillo-Avendaño et al., 2018). Both naturally occurring environments and tissue culture settings can support this type of *de novo* root or shoot regeneration. (Xu and Huang, 2014; DeClercq et al., 2011)

Naturally during *de novo* root regeneration, the damaged plant organs or tissue grow new roots (DNRR). DNRR may happen in several plant organs, but out of all these organs, the leaves respond in a particularly interesting way: if a leaf is cut from the plant and allowed to develop in its natural environment, it will sprout new roots on the side that was cut. (Figure 3) (Shanmukhan *et al.*, 2021).

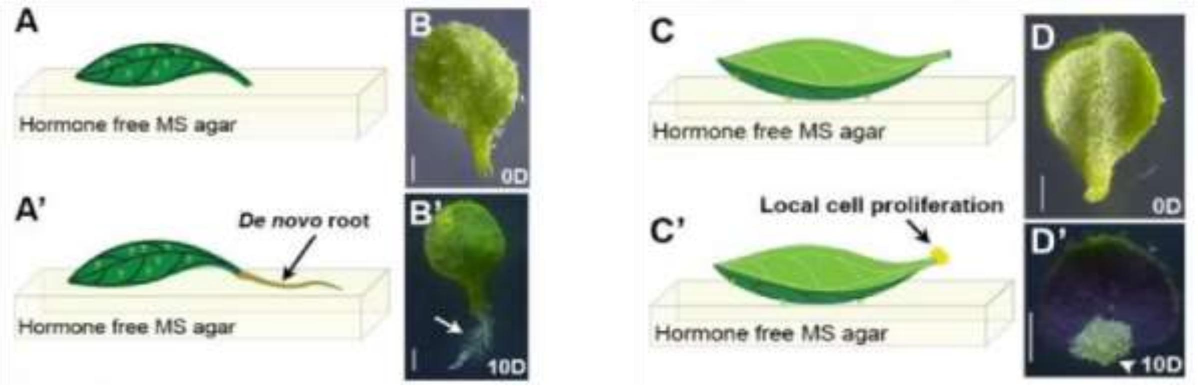


Figure.4: Regenerative response at the cut end in the *A. thaliana*. DNRR response from the cut part of the leaf in the existence of touch scenario (A, A', B, B'). And the formation of callus when the leaf is placed on the MS-agar media so that the cut part is not touching to the media (C, C', D, D'). 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). This type of regeneration can be distinguished by the fact that there is no application of phytohormones exogenously as is 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.

The DNRR responses in the hormone-free medium happens at the sharp cut end of the *Arabidopsis thaliana* leaf. However, another different response to the injured cut ends of model plant organism *Arabidopsis thaliana* leaves is the development of callus, which is a sign of wound healing (Shanmukhan et al., 2021). The question here is what decides the outcome of regeneration (de novo root vs. callus). Actual physical contact between the cut end and a solid or liquid surface influences the decision to develop the DNRR over the callus. The leaf explant forms *de novo* roots when the cut end of the leaves is positioned in touch with the hormone less medium surface (Shanmukhan et al., 2021). On the contrary, the leaves displayed a formation of callus which is considered as wound-healing response when leaves were placed such that the cut end was not in contact with the medium (that is, in the air) (Figure 4). The authors also examined the auxin level in detached leaf. When the sliced portion of the leaf comes into touch with the agar medium, the auxin level is higher than it is in the leaf where the cut piece does not come into contact with the ½ MS agar media's surface leading to no interaction with surface (Shanmukhan et al., 2021). This indicates that the DNRR response involves Auxin (Pop *et al.*, 2011; Xu, 2018; Mathew and Prasad, 2021).

1.4 Cellular and Molecular Foundation of DNRR

A. **Cellular Basis -** During the de novo organogenesis process, a cell has to repair itself and initiate a survival strategy. Here, the cells have to adopt the identity of the new organ by reprogramming themselves. In order to do this, the original cells that possess the ability to regenerate must go through dedifferentiation to attain the status of stem cells. Then, they could change into cells that vary from the parent cell—in this case, root cells. According to da Rocha Correa et al. (2012) and Chen et al. (2016), adult stem cells from aerial organ vascular tissue, such as procambium or cambium cells, encourage the formation of new roots. For de novo organogenesis to occur, the cells must proliferate after cellular dedifferentiation and competence. Ultimately, the cells need to take on a new cell destiny, which is accomplished by gene regulation unique to the cell type and hormone reactions.

B. **Molecular Basis -** The phytohormones such as auxin and cytokinin are essential in determining whether roots or shoots will regenerate. A high ratio of auxin to cytokinin encourages the growth of roots, whereas a high ratio of cytokinin to auxin encourages the growth of shoots (Ikeuchi et al., 2019). For the DNRR response to occur, auxin is essential (Pop et al., 2011; Xu, 2018; Mathew and Prasad, 2021). According to research (Sun et al., 2016), root regeneration is not observed in explants grown in environments that contain auxin biosynthesis inhibitors or auxin polar transport inhibitors. Auxin transporters PIN-FORMED 1 (PIN1), PIN2, PIN3, and AUXIN RESISTANT 1 (AUX1) are associated with the development of DNRR. (Braybrook and Harada, 2008). Additionally, auxin accumulation activates WUSCHEL-RELATED HOMEOBOX (WOX11, WOX12, WOX5, and WOX7) and AUXIN REPONSE FACTOR (ARFs), which aid in the acquisition of a new destiny for cells (Liu et al., 2014; Chen et al., 2016). Therefore, the production and polar transit of auxin are essential for the DNRR response.

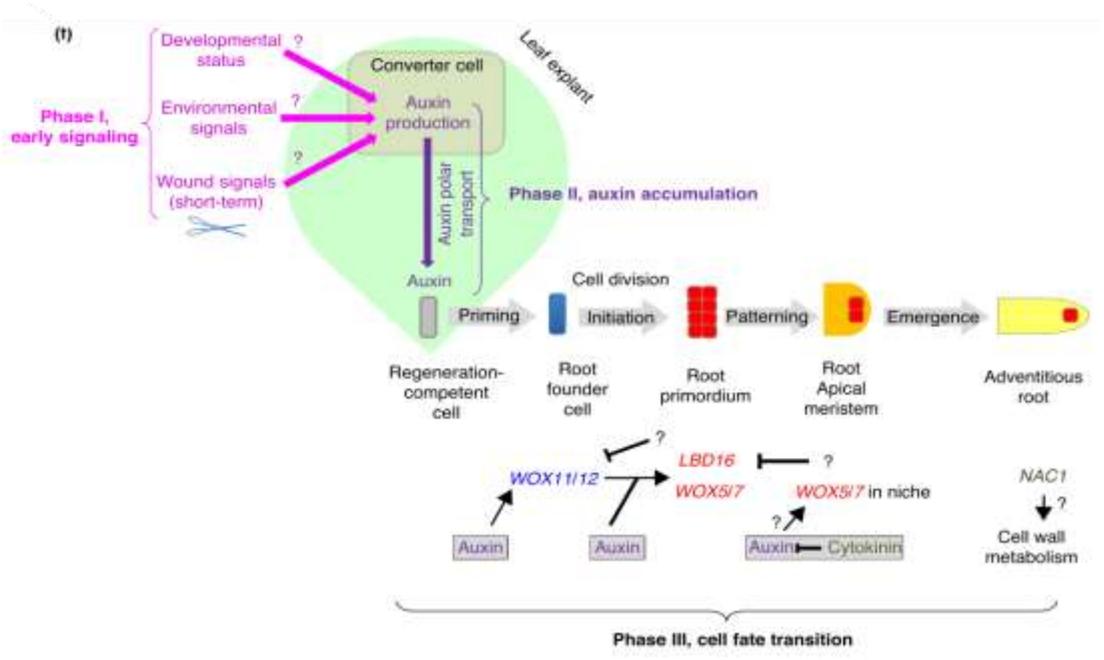


Figure 5: Cellular and Molecular framework of DNRR. Adapted from (Xu, 2018)

C. **Three phases of DNRR-** The DNRR's cellular and molecular architecture was broken down into three stages by author Lin Xu in 2018 (Figure 5). Early signalling, which is mediated by injury, makes up the first step. The detached explant is sensitive to several stimuli, including signals from the wound and surroundings.

Auxin is produced by the cells that receive early signals as input and transmit them to the converter cells from the wound site. Other short- and long-term wound stimuli may also affect this signal transmission process (Xu, 2018). The buildup of auxin takes place during the second phase. The procambium and cambium cells, which undergo reprogramming and produce new roots, collect auxin. A shift in cell destiny is the last phase. There are four more sections to this phase. Transforming regeneration-competent cells into root founder cells is the initial step. In the second stage, root founder cells divide to produce the root primordium. The third stage occurs when root primordium cells appear, giving rise to the root apical meristem. The process culminates with the root tip emerging from the layer of meristem cells. (Figure 5).

1.5 Leaf vein regeneration (LVR)

Leaves are the most crucial photosynthetic organs in plants and are thus immensely important for growth, biomass accumulation and transportation of photosynthates via the vascular bundles. But often they get damaged leading to disruption of flow of photosynthates, and as a result, the leaf's and its neighbouring branch's development is hindered (Radhakrishnan et al. 2020; Sachs and Hassidim 1996). Thus, to maintain continuity of flow, they show different regenerative process. The molecular mechanisms enabling the regeneration of growing Arabidopsis leaves have been investigated using injuries that mimic natural mechanical damages, including leaf vascular injury and surface abrasion (Radhakrishnan et al., 2020).

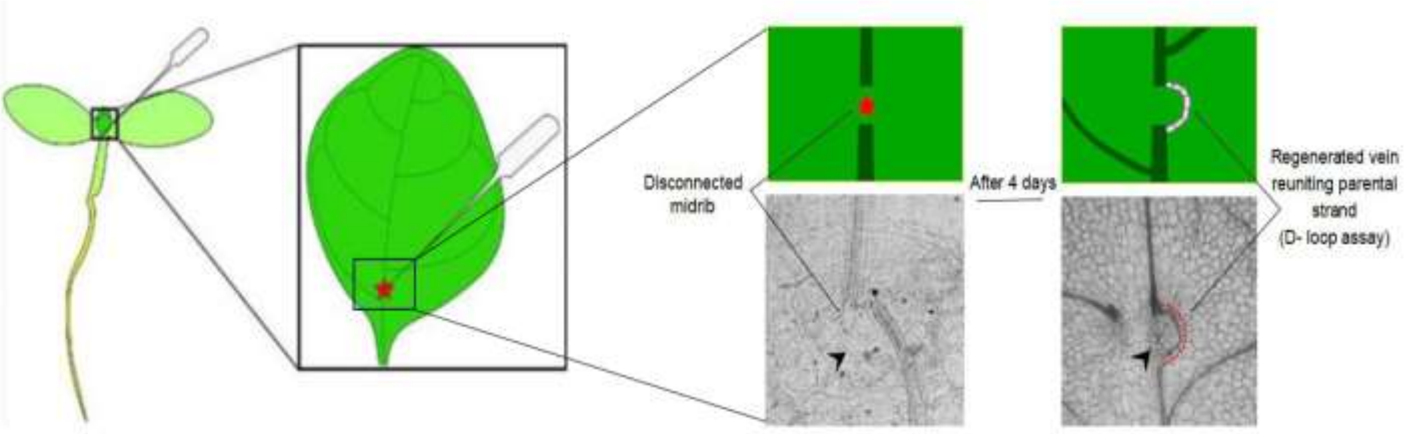


Figure 6. Schematic representing leaf vein regeneration assay. Red star indicates the site of injury in 4 days old seedling of Col-0 wild type. The damage is healed after four days in the shape of a D-loop which forms among the broken injured strands. (Radhakrishnan et al., 2021).

The test for leaf vein regeneration is used to investigate how an injury affects tissue restoration and wound healing. In LVR assays, the incision is done at the basal end of the leaf lamina and between the petiole, or leaf stalk (Fig. 5). This is done so as to make sure that injury has been made rightly at the point which is just above the leaf's first lateral vein where the leaf vein regeneration efficiency is highest as compared to the other different positions across the proximo-distal midvein axis (Radhakrishnan et al. 2020). The abaxial surface (lower surface of dorsoventral leaf) of the leaf pertaining to the initial pair of rosette leaves should be the site of this incisional lesion. During the assay, when the parental stands were disconnected for the 4-day old seedling of Col-0 wild type and upon checking 4-6 days post injury the strands were reunited by regenerating or reuniting the tissues of vascular strands that bypassed the position of injury forming the D-loop structure. These experiments will contribute to our knowledge of the molecular process behind vascular regeneration in developing aerial organs. Following damage, a well-organized feedback loop consisting of PLETHORA (PLT) and CUP-SHAPED COTYLEDON2 (CUC2), which determine cell fate, triggers the production of auxin locally, resulting in the regeneration of blood vessels in developing aerial organs (Radhakrishnan et al. 2020).

Radhakrishnan et al., 2021, showed that leaf vein regeneration is regulated by three different parameters which include injury's size, the plant's age and the location of the damage (Figure 4). Before this study, most of the people believed that local damage to a developing leaf could not be healed. Interestingly enough, all three of these factors have a significant impact on the regeneration procedure.

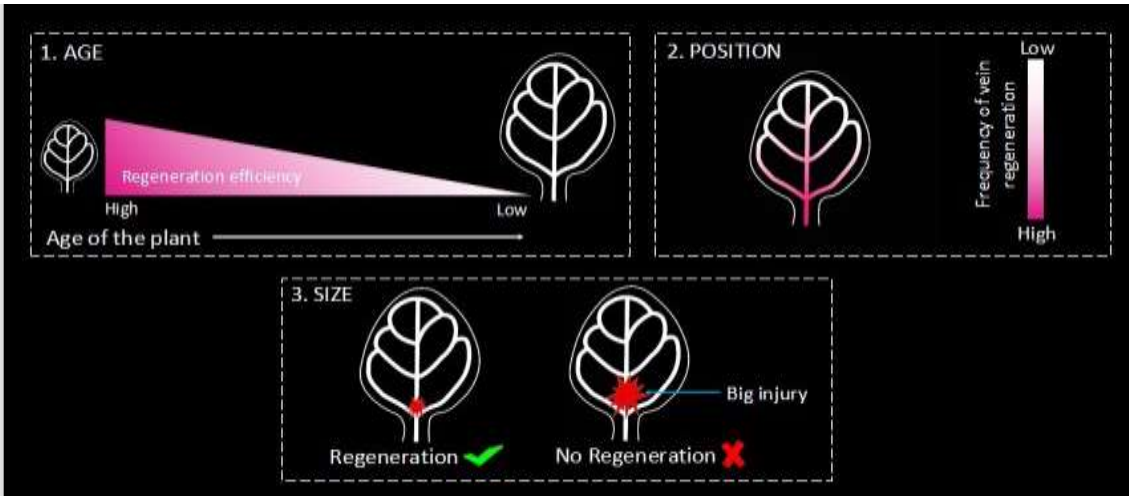


Figure 7. Schematic representation of factors affecting leaf vein regeneration. (1) As plants get older, their midvein regeneration efficiency declines. (2) At the base of the midvein, the vascular strand's regeneration efficacy along the leaf lamina is at its highest. The distal end of the midvein and lateral veins exhibit lower frequency of regeneration. (3) The regeneration efficiency is governed by the size of the injury. It has been observed that injuries bigger than 400$^{\mu}$m cannot be repaired (Radhakrishnan et al., 2021).

2 Motivation for the project;

After discussing the above mechanisms of the DNRR and LVR, there are a lot of unanswered questions which needs to investigated. 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? Why does vascular strand form the D-loop at the site of injury even after healing properly because plants have to synthesize more cellular infrastructure to bypass the wound which is more energetically expensive process.

Based upon DNRR response a hypothesis is present stating that in case of contact-mediated *de novo* root regeneration "Touch to the surface likely enable inhibitors of DNRR to leach out into the media, which would have otherwise accumulated at the cut end hindering DNRR". (Shanmukhan *et al.*, 2021).

Now, one of the common things which unites different types of regeneration and different assays performed is the induction of stress. This is because in different assays either the plant organs are damaged or they are perturbed from the original development path. Due to induction of stress situations, it leads to one of the responses being production of ROS (reactive oxygen species). Previously the inhibitors which was discussed in the hypothesis is speculated to be ROS. ROS can be defined as the partially reduced or oxidised forms of elemental oxygen. It includes singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) etc. (Figure 5). Now, for a very long time ROS have been seen as an unwanted byproduct of aerobic metabolism inside the cells because it causes cellular oxidations leading to disruption of normal physiological functions (Imlay, 2003, 2008; Ogilby, 2010), but over the time literature

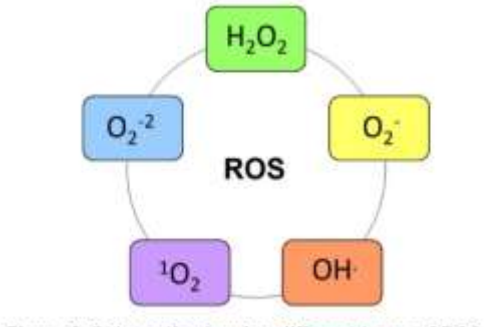


Figure 8. Schematic showing different types of ROS.

have shown the role of ROS to be functional in molecular signalling and in various physiological processes, including in regeneration as well (4 -12).

ROS can be majorly produced in abiotic condition but also in biotic stress as well. But in recent years, a novel theory has surfaced, according to which regulated ROS generation serves as a signal and plays significant signalling functions throughout stress reactions and development (Shapiguzov, Alexey et al., 2012,). Plant cells produce ROS in a variety of compartments. The primary source of ROS in animal cells is mitochondria (Marchi et al., 2012); however, the contribution of all these different organelles to the creation of ROS in the species like plants is less clear (Dutilleul et al., 2003; Suzuki et al., 2012). In addition to mitochondria, ROS are also created in the nucleus and the endoplasmic reticulum, as well as in the chloroplasts, peroxisomes, and apoplast. ROS have the potential to enter cells through plasma membrane channels like aquaporins. This might lead to alterations in gene expression through intracellular signalling pathways, which in turn could enhance the expression of genes with specific functions, such as the Rboh gene family, ZAT gene family, SOS2, PR5, and so on. Out of all these families, the primary stress markers whose induction is a distinct marker of stress are primarily the plant-specific zinc (Zn) finger transcription factor family (C2H2-type) and rboh, which comprises around 20 members of the ZAT family. According to a genome-wide investigation of the Arabidopsis ZFP protein, ZAT members have been demonstrated to be involved in a variety of cellular activities, including as oxidative stress responses, seed germination, drought, cold, and high salinity (Li, Hao et al., 2022).

3 Goal of the project

The primary objective of this study is to explore *de novo* root organogenesis (DNRR) and Leaf vein regeneration (LVR) assay.

Aim 1- To investigate the variation in regenerative responses from the *rbohD* and *rbohF* mutants ordered from the ABRC (Arabidopsis biological resource centre).

Objective 1.1- To perform a DNRR and LVR assay on *Arabidopsis thaliana* to check the regeneration responses of different mutants.

Aim 2- With the help of ROS responsive fluorescent transcriptional reporter consisting the promoter of transcription factor ZAT12 to drive the expression of free mCherry (Red fluorescence protein), in order to understand the fluctuations in the dynamics of ROS productions.

Objective 2.1- To observe spatiotemporal dynamics of the ROS response during de novo root regeneration (DNRR).

Objective 2.2- To investigate cell-to-cell behaviour during stress response for regeneration.

Chapter 2 Materials and Methods

2.1 Plant materials:

All of the plant species taken into consideration for the study are of *Arabidopsis thaliana* having Columbia as an ecotype. Different transgenic and mutants of Arabidopsis were used in floral dip transformation for transforming the ROS responsive construct *(pFRm43GW (spec, RFP) ZAT12::mCherry: NosT)* which includes: -

- **Cell cycle marker** - *Col; pg CDT1α-YFP, Col; pg CYCB1-GFP, Col; pg RBR-GFP*
- **Cell membrane marker** – *Col: pUBQ10::td Tomato 29-1*
- **Auxin Sensor** – *DR5rev::3XVenus-NLS*
- **Transcription Factor** – *Col; pg PLT7-vYFP* (Transgenic),
- **Mutant** - *plt3,5,7*
- **Microtubule marker** – *Col YFP CLASP*

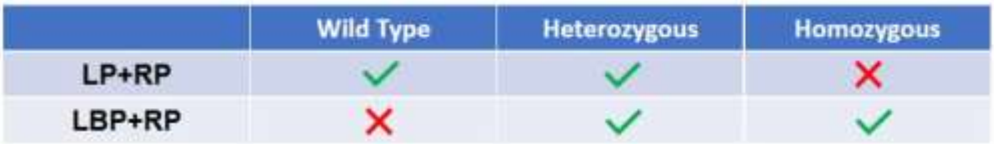
AtrbohD and *AtrbohF* mutants were also used to study effect of these mutants on the various regeneration assay. These mutants were ordered from the **ABRC (Arabidopsis Biological Research Centre)** and have been created with the help T-DNA insertion.

2.2 Seed sterilization and growth conditions

For the seed surface sterilization of Arabidopsis thaliana, a Seventy percent ethanol wash, twenty percent bleach wash followed by 7 times autoclaved distilled water wash was given. Sterilized seeds went under the 3 days vernalization (Cold treatment) to break their dormancy. For germination, seeds were plated on ½ strength Murashige-Skoog (MS) media having agar as a solidifying component with pH range of 5.70 – 5.72 and the plates were positioned vertically in plant growth chamber giving them the suitable conditions for their growth which includes temperature of 22°C, under 45µmol/m^2/s white light and 70% relative humidity. One square Petri plate (120mm*120mm, Himedia model: PW050-1) with roughly 50mL half-MS media is used to plate 20 to 30 seeds.

2.3 Genotyping (PCR):

To screen the *rboh* mutant for their homozygous or heterozygous nature of mutant various combination of primers were used. This technique is called Genotyping. Genotyping is defined as a way to know the differences in genetic make-up of an organisms by examining the genome sequences using various biological assays. In this case we will PCRs as a method to assay the genome. Also, genotyping is done so as to screen out homozygous mutants from the heterozygous mutants because heterozygosity creates redundancy which will hinder our goal of seeing the effect of mutants on our interest of physiological process. Mutants for Rbohs is created with the help of T-DNA insertion and to screen out the homozygous mutants, PCRs were done.



	Wild Type	Heterozygous	Homozygous
LP+RP	✓	✓	✗
LBP+RP	✗	✓	✓

Table-1: Possibility of result upon genotyping using various combination of primers

Upon using the combinations of LP and RP (Left primer and right primer of Rboh) and LBP (Binding site of primer on T-DNA) we were able to segregate the different mutants. We get to see the band in case of wild type when LP+RP is used but no when LBP+RP. This is because when a T-DNA is added, it mutates the whole structure of gene by increasing its overall size. Thus, when PCR is done where extension time of polymerization is given with respect to the non-mutant form of gene and giving us a conclusion of positive control (wild type) is working. Similarly, when PCRs is run for heterozygous mutants, it will show the bands on gel in both the conditions i.e., LP+RP and LBP+RP. This is because in a pair allele of a gene only one is mutated. And in case of homozygous as whole allele is mutated, we get to see band in LBP+RP only.

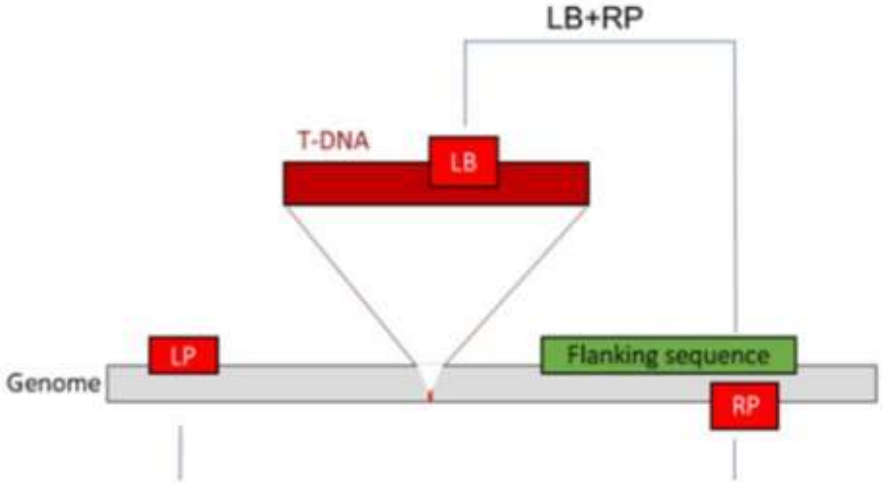


Figure 9. Schematic representation of T-DNA insertion. The results of the screening process using the three primers (LB+LP+RP) should show that the wild type (WT) has no insertion in it and should produce a product from LP to RP; the homozygous lines (HM) have insertions in both chromosomes and will produce a band from RP to LB, to the left border of the vector; and the heterozygous lines (HZ) have insertions in one of the pair chromosomes. (Batth et.al., 2020)

Following set of primers were used for genotyping the rbohD and rbohF mutants to screen out the homozygous.

Sr. No.	Gene Primer	LP	RP
1.	AtrbohD	5’-TTTCAACGCCTTTTGGTACAC-3’	5’-GTTACCTATTCTTTTGCCGGG-3’
2.	AtrbohF	5’-CAAAGAGCTCTTCGTGGTTTG-3’	5’-TCTCTATTGTATCTTGTGTCACCG-3’

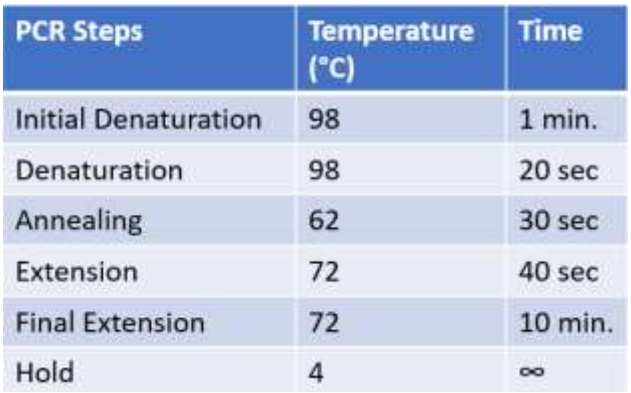
Table-2: List of Primers for *rbohD* and *rbohF* screening.

2.4 Genomic DNA extraction:

Utilizing the CTAB (cetyltrimethylammonium bromide) reagent in accordance with the protocol instructions (2), the DNA was extracted from the leaf tissue. This genomic DNA was used for genotyping (Process of finding differences in genetic make-up of an organisms using various biological which includes PCR) of *rboh* mutant.

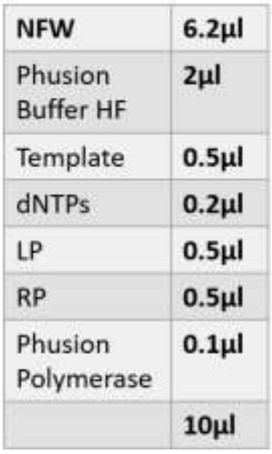
2.5 Thermocycler Program and Reaction Mixture

Following condition PCR and reaction mixture were used to screen out the homozygous mutant from the population containing heterozygous mutant and Wild type mutants.



PCR Steps	Temperature (°C)	Time
Initial Denaturation	98	1 min.
Denaturation	98	20 sec
Annealing	62	30 sec
Extension	72	40 sec
Final Extension	72	10 min.
Hold	4	∞

2(a)



NFW	6.2µl
Phusion Buffer HF	2µl
Template	0.5µl
dNTPs	0.2µl
LP	0.5µl
RP	0.5µl
Phusion Polymerase	0.1µl
	10µl

2(b)

Table-3: 3(a) Thermocycler condition. 3(b) Reaction mixture

2.6 Multisite gateway cloning

The cloning used here was Multisite Gateway cloning which works on principle that is being used by bacteriophage (Virus particle) to infect the bacteria.

PRINCIPLE: - Multisite Gateway cloning relies on the principle of site-specific homologous recombination machinery used by bacteriophage called Lambda phage which integrate its genome into the host genome (*E coli*). Below schematic figure depicts the integration system involving attP-sites and attB-sites. Recombination between the two attB and attP elements giving rise to new recombination sequences called attL sites and attR sites which flanks the inserted phage DNA. Since Gateway Cloning is all about transferring elements from one plasmid to another plasmid, these are referred to as **BP reaction** (BP→LR) and the **LR reaction** (LR→ BP) rather than using the terminology such as integration and excision.

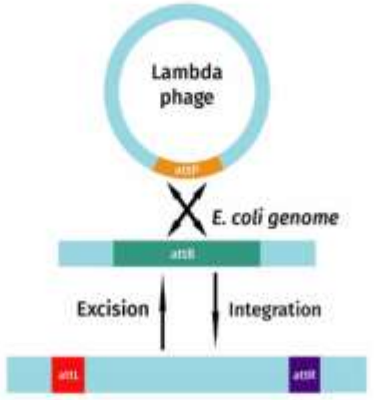


Figure 10. Schematic of (λ)Lambda Phage strategy used in multisite gateway cloning.

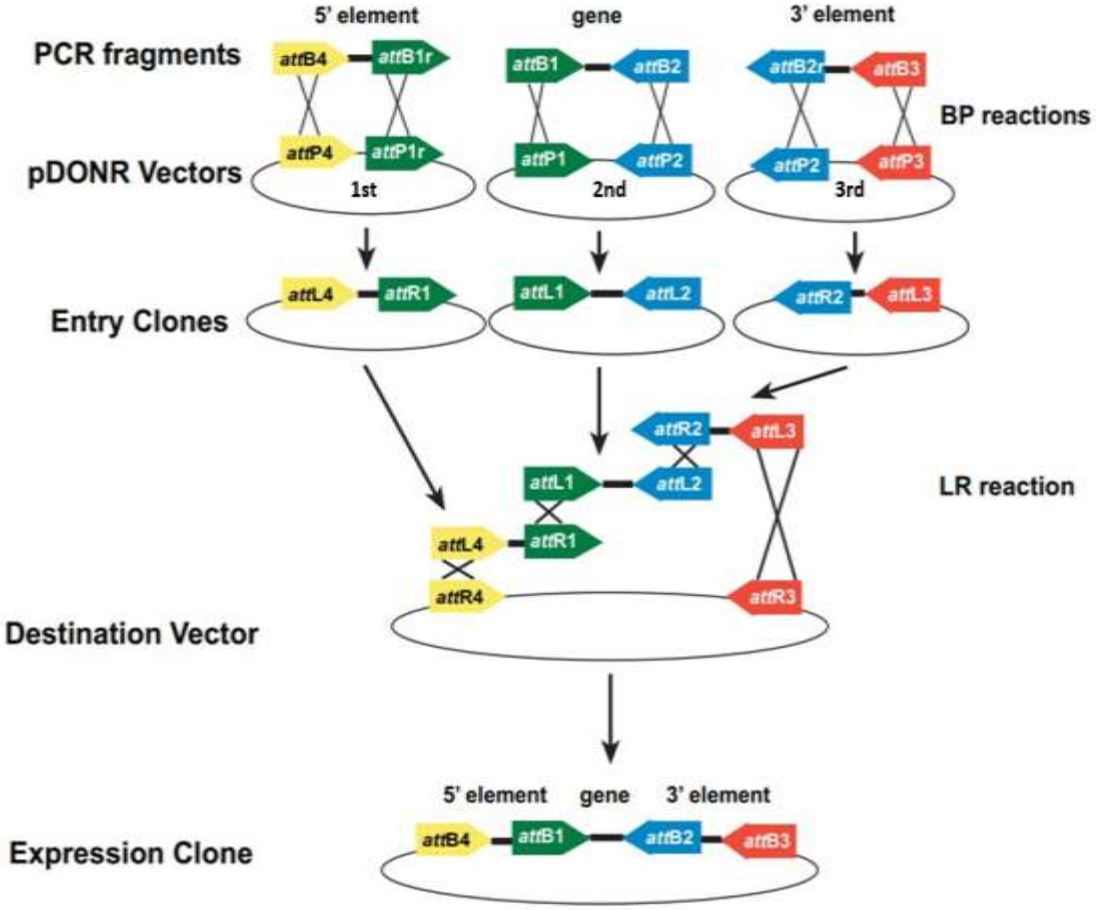


Figure 11. Schematic representing overall Multisite Gateway cloning procedure. The entry clone containing the DNA of interest flanked by attL and attR sites is produced by the BP Reaction between the attB sites surrounding the insert and the attP sites of the donor vector. The created entry clone's attL and attR sites, as well as the destination vector's attR sites, engage in the LR Reaction. resulting in the creation of an expression clone that has attB sites surrounding the desired DNA. Many fragments (in this example, three fragments) can be combined by multisite gateway cloning into a single destination vector that is bordered by att sites. (**Invitrogen**)

To perform Multisite Gateway cloning, choosing the correct order of pDONR vector is essential meaning every pDONR has been designed in specific way which ends up contributing to either the 5' or 3' element or middle part of our construct. For example, 1st pDONR vector has modified att sites which allows to it form only 5' part of construct similarly, the 2nd and 3rd pDONR vectors contribute to the middle and 3' end of constructs only. First BP reaction is carried out and eventually after confirming every BP reaction via digestion the LR is set up. The entering plasmid clones which are used in the LR reaction must align correctly with respect to position. Prior to their recombination, the CcdB gene was already present in the donor and destination vectors. During their respective BP or LR reactions, it is exchanged with the gene of interest. The majority of colonies should include the desired gene of interest, recombined construct, as the CcdB gene prevents the production of DNA gyrase, inhibiting the development of E. coli strains that carry it.

2.7 Cloning Elements:

For developing the ROS responsive fluorescent Bio-transcriptional reporter the promoter of ROS-responsive transcription factor *pZAT12* is used to drive the expression of free mCherry (Red fluorescence protein).

Q1) Why pZAT12 was used as a promoter?

1) The AtZAT12 promoter responds to abiotic stressors such injury with a high degree of selectivity to cellular ROS like H2O2, singlet oxygen, etc. (Miller et al. 2009).
2) When compared to the promoters of other ZAT members that get active under stress, the AtZAT12 promoter system seems to have a better reaction time and sensitivity. Under the direction of ZAT12 promoters, the ZAT12p-ROS bioreporter, a quantitative ROS transcription-based bioreporter, exhibits a fast rise in ROS response as early as 5 minutes after plants are exposed to challenges such as oxidative stress, salt stress, and pathogen defence (8).

3) Rapid induction of stress-related molecular responses was shown to be highly connected with the stress-associated alterations in ROS response as reported by the Zat12p-ROS bioreporter, according to qPCR analysis. (8).

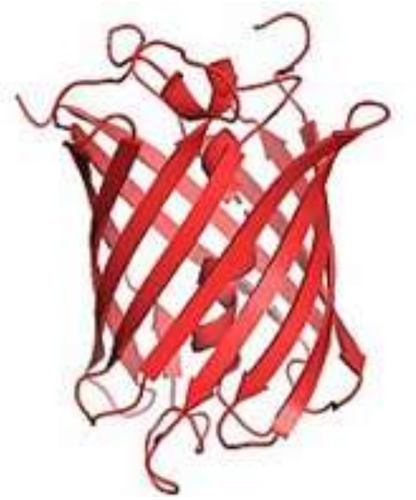


Figure 12. Protein structure of mCherry (Wikipedia)

Q2) Why was mCherry was used as a reporter?

1) The pKa value of mCherry is 4.5 (9). This value is obtained from the amino acid present on the chromophore located inside the barrel shaped protein and along with some additional amino acids on the mCherry protein structure. A fluorescent protein's acid sensitivity is gauged by its pKa value. Additionally, it is the pH at which fluorescent protein fluorescence intensity decreases to 50% of its

maximal value(pH=pKa). Thus, at any point of time the pH of cell ranges from pH (5) – pH (8), giving us the assurance of its functional efficiency fluctuating between 60-90%.

2) Maturation time of mCherry is 15min (9). Meaning it is the time required by the protein to fold properly and allowing its chromophore to mature for fluorescence to obtain half-maximal value.

3) The fluorescence lifetime of mCherry is 1.4ns (Nanosecond) (9). Meaning the moment at which the ZAT12 promoter stops firing, we can expect that in a reasonable time frame mCherry expression will go off giving us the exact reliable ROS read out. In other words, we can trace the exact time span at which cell is experiencing stress.

Q3) Why was NosT was used as a terminator?

1) **1)** The polyadenylation (poly-A) signal included in the NOS terminator is helpful in adding a poly-A tail to the mRNA, which makes it more stable and effective during translation in eukaryotic cells, such as plant cells. (Depicker, A et al. “Nopaline synthase: transcript mapping and DNA sequence.” *Journal of molecular and applied genetics* vol. 1,6 (1982): 561-73.)

2) The NOS terminator, being derived from Agrobacterium, is compatible with the Agrobacterium-mediated transformation process, ensuring efficient gene transfer into plant cells.

2.8 Multisite Gateway cloning Overview

Firstly, the PCRs are carried out incorporating the attB sites flanking gene of interest. Followed by carrying out BP reaction. Now, in BP reaction the pDONR vectors having attP sites along with the gene of interest which is having attB sites is added to a small Eppendorf facilitated by BP Clonase enzyme and NFW (Nuclease free water). This BP reaction is carried out for 6-8 hours at 25°C. This reaction is stopped by adding proteinase K enzyme. This reaction mixture is transformed into bacteria (*E. coli*) with help of heat shock method. Following this, each colony will be subjected to a positive selection screening process. Upon confirmation the last and final step of Multi site gateway can be carried out which is LR reaction. Now, in LR reaction the multiple fragments are added having attL sites along with the destination vector which is having attR sites is added to a small Eppendorf facilitated by LR Clonase enzyme and NFW (Nuclease free water). This LR reaction is carried out for 16 hours at 25°C. The same after procedure is followed for screening the colonies obtained from BP reaction for the LR reaction as well. Upon confirmation the construct is transformed into *Agrobacterium tumefaciens* (C58) with help of electroporation method. Finally, the Agrobacterium is also screened for positive with help of PCRs and upon confirmation the floral dip method is carried to introduce gene of interest in plant.

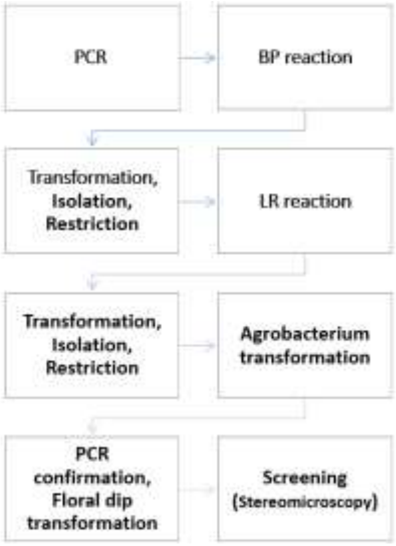


Table-4: Workflow of the multisite gateway cloning.

2.9 Floral dip (Modified)

One common approach for inserting foreign DNA into the *Arabidopsis thaliana* plant genome is the floral dip method. 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 C58 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 containing LB broth as a source of nutrients and then supplemented with suitable antibiotics for bacterial selection and then incubate at 28 to 29°C for 24 hours if it is a primary culture and followed by 16hours if it is a secondary culture. 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 now infected with the Agrobacterium suspension culture containing the surfactant with the help of pipette or droppers by adding the culture on to the plant drop by drop making sure the drops remain suspended on the floral buds. Here, the surfactant helps the bacterial suspension stick to the floral tissues. Now, cover the treated plant with the black plastic cover to maintain high humidity for 16-24 hours. Shift the plant to the growth chamber to mature. Protect the treated plant from high temperature and excessive light to prevent them from heating up. If the flowers successfully turn into siliques (seed pods), the seeds within them might end up transmitting the injected DNA. Transformed seeds are extracted and are screened for transformed seeds from the population of non-transformed seeds. This screening can be done either with help of antibiotic selection media where only the transformed grows properly because of the presence of construct within them or with the help of seed coat selection marker in which transformed seeds shines up under the fluorescence stereo zoom microscope. 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.

2.10 Fluorescent dye staining

Under both normal or pathological circumstances, oxidative stress is an essential phenomenon. Therefore, it is crucial to use fluorescence dye to measure total reactive oxygen species production (ROS) in order to assess oxidative stress. The most common dye used is 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA OR H_2DCFDA) for such purpose. This staining procedure is quick and affordable to detect

ROS at an any point of time with a cost-effective management. In the procedure H_2DCFDA is loaded onto to the plant specimen at a concentration of 1 μM with an incubation time of approximately 5 – 10 minutes for seeing the expression followed by wet mounting (procedure to suspend the specimen in the any liquid medium between the coverslip and glass slide) it with water for seeking better quality of images (39). This staining can be applied to the detection of ROS production for internal ROS production like H_2O_2 upon genetic or chemical alteration to an organism. As a result, it is beneficial to pinpoint cellular oxidative stress as a consequence to various different sorts of stress, which offers hints for mechanistic investigations.

2.11 Microscopy and PCR gels

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

2.12 Statistical analysis

The statistical analysis of images was analysed with the help of the Fiji program (Schindelin *et al.*, 2012). Furthermore, the data is plotted and analysed in software called GraphPad Prism version 9.5.1 software (www.graphpad.com).

Chapter 3 Results

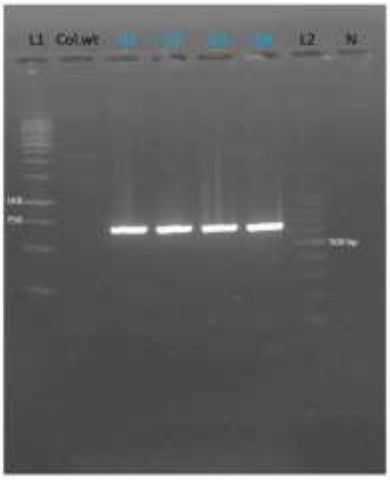
Section 3.1 Regenerative responses in mutants and wild type species of *Arabidopsis thaliana*.

3.1.1 Screening for the homozygous mutants.

According to Torres and Dangl (2005), Rbohs are the plant homologs of the NADPH oxidase which is found in mammalian phagocyte having common component gp91phox. There is total ten members Rboh genes family found in the Arabidopsis thaliana genome. It was discovered that the two main NADPH oxidases expressed in all over the plant body were AtRbohD and AtRbohF (Miller et., al 2008). Thus, to see the effects of mutants *rbohD* and *rbohF* on the *de novo* root regeneration, we ended up doing the genotyping of mutants to screen for their homozygosity (Explanation of genotyping in Section-3.5).

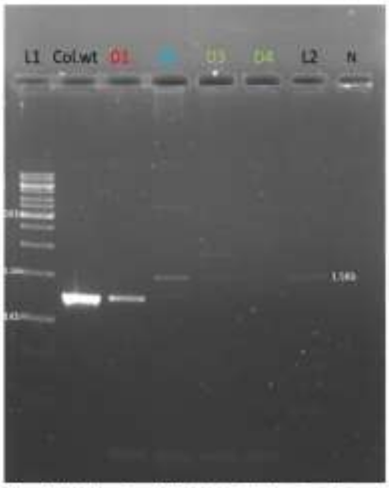
From the figure **12(A)** and **12(B)** it is observed that when the PCR products were run for LBP+RP and LP+RP, there was no band observed in case of Columbia wild type for LBP+RP as oppose to that there was band observed in case of LP+RP (Expected product size = 1248 bp) indicating that Columbia wild type which is considered as positive control is working. In both the gels the negative control (N) had no band giving significance PCR reaction is being specific and have amplified what is to be amplified. Similarly, when the different *rbohD* samples (D1– D4) went under the same screening process, D3 and D4 sample stood out to be positive for being homozygous because

(A)



L = ladder (1kb), L2 = ladder (500bp), N = Negative control, D = *rbohD*
Expected LBP+RP Product Size range = 577-877 bp
Conclusion :- Positive control (Col WT) is working

(B)



L1 = 1Kb ladder , L2 = 100bp ladder, N = Negative Control
Expected LP + BP Product Size = 1248 bp
Conclusion :- D1 = Heterozygous, D3 & D4 = Homozygous

band was only observed in case of LBP+RP (product size range = 577 to 877 bp) and not for LP+RP. D1 is heterozygous because band is seen in both the cases.

Figure 13: PCR analysis of confirmatory PCR products after the genotyping of *rbohD* mutant. Out of the 4 samples of *rbohD*, D3 and D4 are homozygous. PCR products were separated on 1% agarose gels.

Similarly, from the **figure 14** it is observed that when the PCR products were run for LBP+RP and LP+RP, there was no band observed in case of Columbia wild type for LBP+RP as oppose to that there was band observed in case of LP+RP (Expected product size = 1172 bp) indicating that Columbia wild type which is considered as positive control is working. In the gel the negative control (N) had no band giving significance PCR reaction is being specific and have amplified what is to be amplified. Similarly, when the different *rbohF* samples (R1– R3) went under the same screening process, R1 sample stood out to be positive for being homozygous because band was only observed in case of LBP+RP (product size range = 544 to 844 bp) and not for LP+RP. R3 is heterozygous because band is seen in both the cases. R2 seems wild type having no T-DNA insertion.

L1 & L3 = ladder (1kb), L2 = ladder (500bp), R = *rbohF* mutants N = Negative control Expected

Expected LP + RP Product Size = 1172bp , Expected LBP + RP Product Size = 544-844bp ,

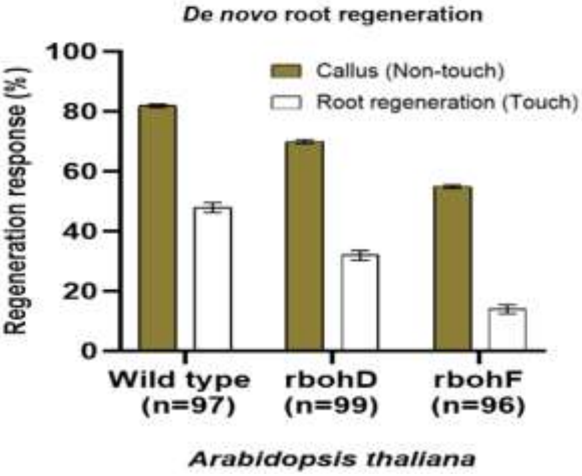
Conclusion = R1 is only homozygous.

Figure 14: PCR analysis of confirmatory PCR products after the genotyping of *rbohF* mutant. Out of the 4 samples of *rbohF*, R1 is homozygous. PCR products were separated on 1% agarose gels.

3.1.2 *De novo* root regeneration (DNRR) Assay

When the homozygous mutant (*rbohD and rbohF*) of Arabidopsis thaliana having Columbia as an ecotype were used up for the *de novo* root regeneration (DNRR) response (Shanmukhan et al., 2021), they showed various types of response. When the sliced cut end of the lateral organ called leaf was kept in touch with the ½ MS media containing sucrose, MS powder and plant agar, these mutants homozygous plants showed similar responses to that of wild type of model plant A. thaliana, i.e., *de novo* root formation (DNRR) but to lesser extent. When the comparisons were done among the wild type and mutants, *rbohD* showed DNRR response to an extent of 30 percent (n = 99) whereas the mutant *rbohF* showed the response to 15 percent (n = 96) when compared to wild type whose root regeneration response was close to 50 percent (n = 97). (Figures 15). Other response includes wound healing and callus formation response at site of cut end when the leaf was kept in non-touch condition. When the leaf samples which were kept in non-touch position, Wild type (Columbia sp.) showed a callus response up to 80 percent. On other hand, similar decreased response was even seen for the mutants which accounted for around 30 percent and 15 percent for *rbohD* and *rbohF* mutants respectively. Other observations include either smoothing of cut surface leading to the wound healing or no response was observed at the site of cut injury due to death of the leaf samples.

(A)



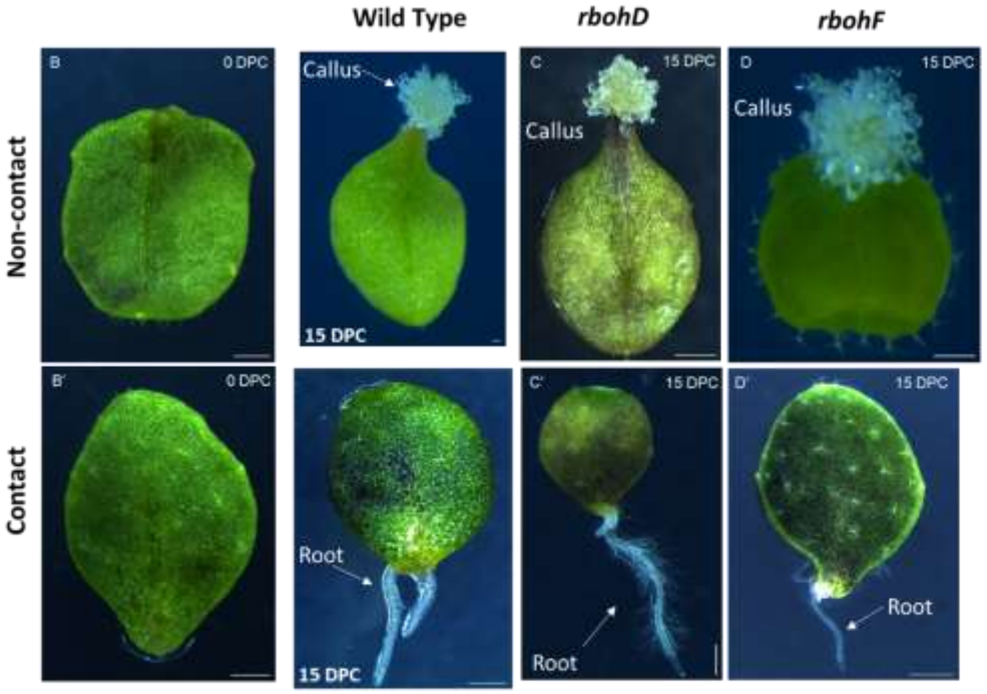
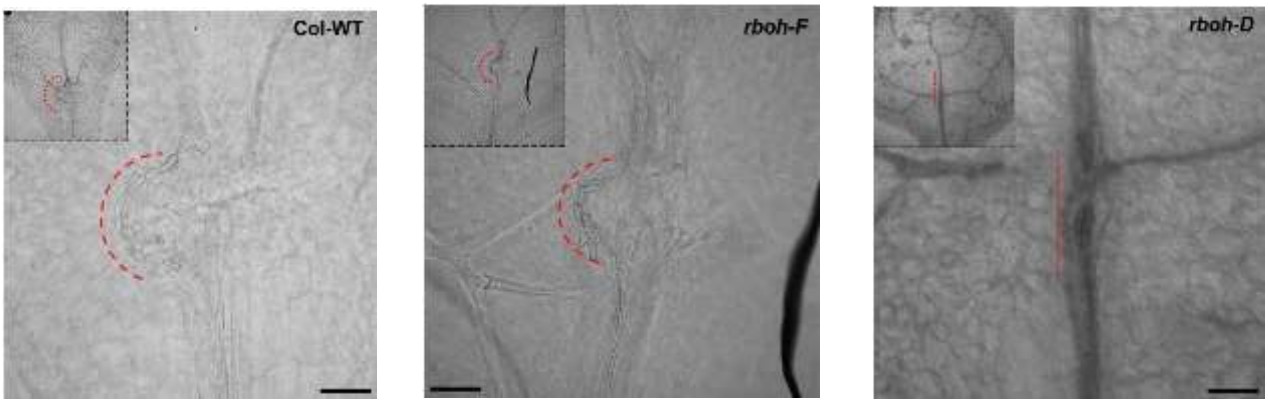


Figure 15. *De novo* root regeneration assay in wild type (Columbia) and mutants. 15(A) Graph showing varying percentage of *de novo* root regeneration between different mutants & wild type. The regeneration response is shown on the X-axis, while A. thaliana samples are shown on the Y-axis.15(B) The detached leaf with the adaxial (upper surface of leaf) side down or cut end not touching to media, resulting in callus development, was captured in stereo zoom microscope images. 15(B') The detached leaf with the abaxial (lower surface of leaf) side down or cut end touching to media, resulting in root development, was captured in stereo zoom microscope images. 15(C and C'), the stereo zoom microscope pictures of the detached leaf with the regenerated *de novo* roots and development of callus of the *rbohD* mutants. 15(D and D'), the stereo zoom microscope pictures of the detached leaf with the regenerated *de novo* roots and development of callus of the *rbohF* mutants. Scale bars: 4mm. Error bar stands for s.e.m. DPC, days post cutting.

3.1.3 Leaf vein regeneration (LVR) Assay

When the phenotype of the *rbohD* and *rbohF* mutants were observed by performing leaf vein regeneration assay along with wild type, as such there was no change observed in the regeneration efficiency as compared to the wild type {Figure 16(B)}, but interestingly there was a change in the path of reunion between physically disconnected strands. In case of Columbia wild type (*Arabidopsis thaliana*) which is

(A)



used as a control, almost all samples showed the D-loop as a path of reunion upon regeneration upon 4 days post Injury (DPI) when kept in continuous light condition. Similarly, under the same condition when the mutants were observed, in case of *rbohD* mutant, half of the sample showed D-loop and half of the sample showed a linear path of reunion. And for the *rbohF* mutant, regenerated sample showed D-loop which was three times the samples showing straight path of reunion {Figure 16(C)}.

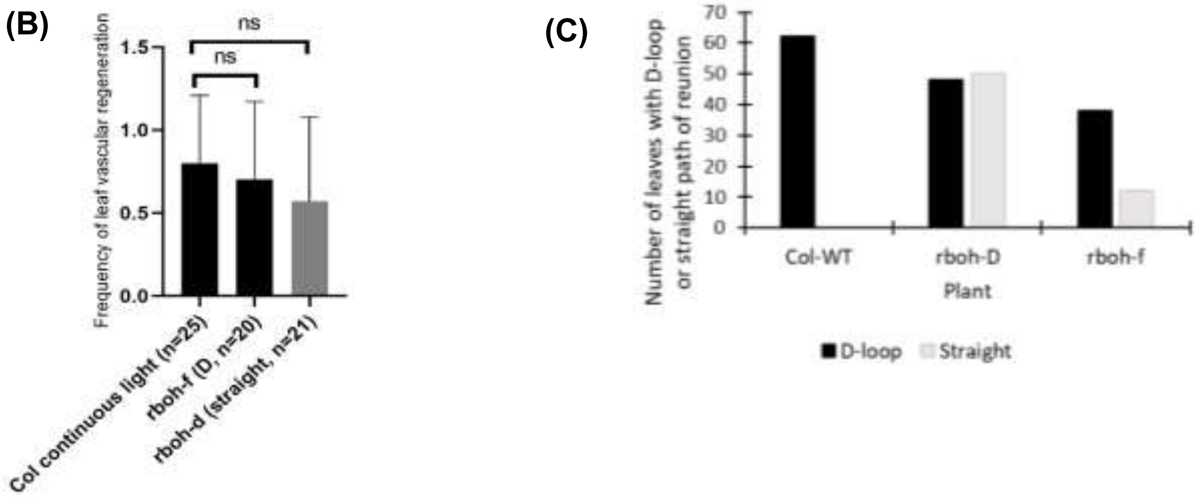


Figure 16. Leaf vein regeneration assay in wild type and mutants. 13(A). Confocal images of cleared transparent leaves showing different paths of reunion of disconnected strands. Continuous exposure of light to injured leaf samples results in the shape of D-loop in case of rbohF and wild type. And linear vein regeneration is found in case of *rbohD.* 13(B) Graph showing frequency of leaf vascular regeneration between different mutants and wild type. 13(C) Graph showing the frequency of D-loop formation versus the straight path of reunion in regenerated samples. Y-axis represents the grey value. Scale bars: 50 µm. Error bar stands for s.e.m.

Now, we were interested to check the correlation between the amount of ROS generated post injury in the mutants and wild type with the samples showing the D-loop versus straight path of the reunion in regenerated samples. This was done with the help of H_2DCFDA fluorescent dye staining.

3.1.4 ROS patterning upon injury

With the help of H_2DCFDA fluorescent dye, ROS quantifications can be done. When done with the different mutants of *Arabidopsis thaliana* which includes *rbohD* and *rbohF*, and wild type then following results were obtained.

Initially when assay was started with time lapse imaging for the Columbia wild type at 5-10MPI (minute post injury) time point, more ROS production was seen at the site of injury (dotted encircled) as compared to the other time points. Furthermore, when we did more time points at the interval of 30 MPI we started seeing less ROS production at site of injury or the cells which are immediately surrounding the site of injury. This ROS production goes on decreasing further on from 24-HPI to 48HPI (hour post injury) which us idea that something might be helping the organ in acclimatizing to the oxidative stress situation. Finally, when expression was seen for the 48-HPI, there was significant decrease in ROS production at the injury's site (dotted encircled).

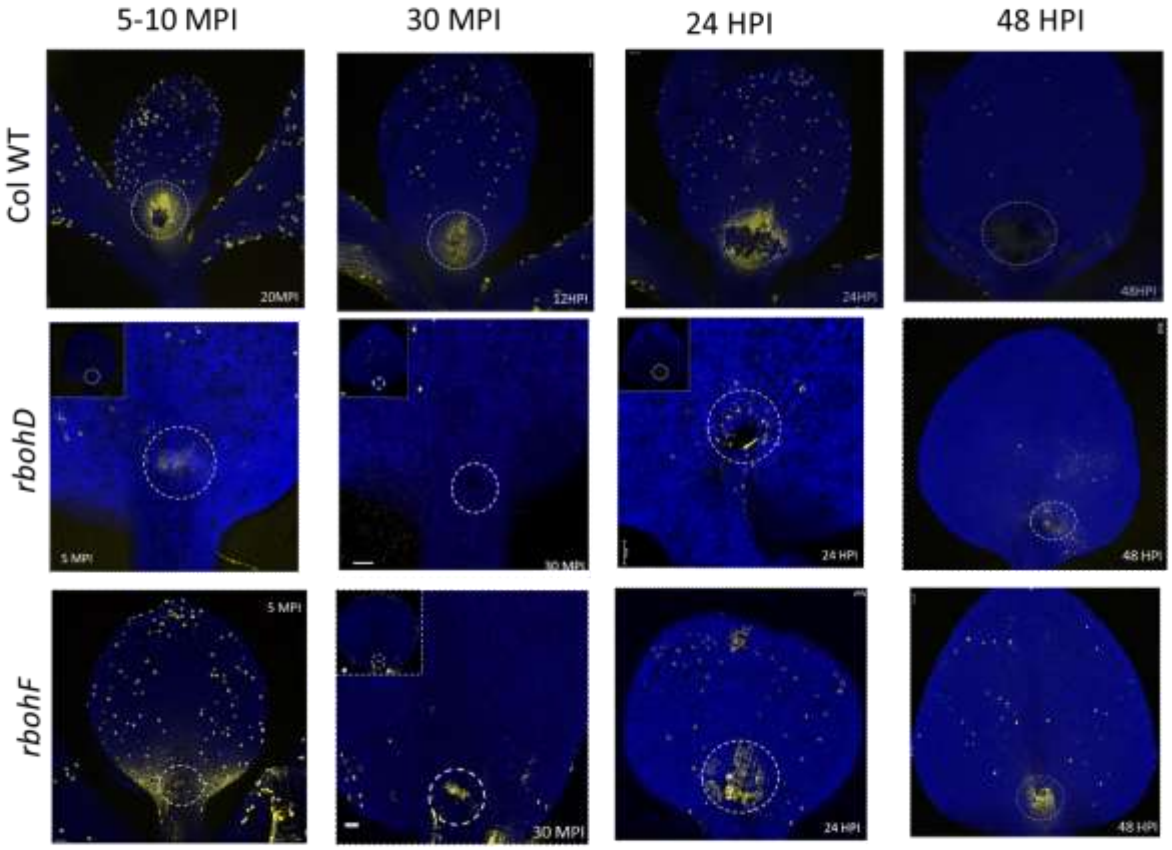


Figure 17. ROS expression pattern during leaf vein regeneration. Time lapse imaging of ROS in injured leaf (dotted encircled) of wild type Columbia (Col WT) with the help of fluorescent dye (DCFHDA). Similarly, confocal imaging of the ROS mutants (*rbohD* and *rbohF*) was captured at various time points. The fluorescence time points imaging includes 5-10MPI, 30MPI, 24HPI and 48HPI. Scale bars: 50 μm

Now, next step was to compare the ROS expression pattern of mutants (*rbohD* & *rbohF*) with the wild type. Starting with the *rbohD* mutant, ROS production was almost nil at the injury's site (dotted encircled) when observed for the 5MPI (minutes post injury) time point. This can be said on the basis of the fact that we are able to see fluorescence in the form of small dots or punkt (yellow dots) which are nothing but the stomata where the ROS production is often more in amount (40) as compared to other regions of leaf lamina. This indicate that staining has been done properly and what we see as expression at the site of injury is not a false result. Furthermore, when we did imaging for 24HPI (Hour post injury) time point we started seeing the ROS production at site of injury or the cells which are immediately surrounding the site of injury but in very low amount as compared to wild type. A very little detectable increase in ROS productions occurs from 24-hour post injury to 48-hour post injury (HPI).

Similarly, when carried out the same staining procedure for *rbohF* mutant at various time point, the overall ROS production was more in amount when compared to the other mutant (*rbohD*) but to lesser extent production is observed when compared with the wild type specimen. The ROS production was very little at the injury's site (dotted encircled) when observed for the 5MPI (minutes post injury) time point. Eventually, the ROS production goes on increasing till the 24HPI from 30MPI time point. And then there is decrease in the ROS production observed at the 48 HPI time point.

From the above observations we saw that there was a severe decline in amount of ROS being formed in case of *rbohD* but which was not in case for the *rbohF mutants* which explains that why we got more of D loop in *rbohF* mutant samples as compared to *rbohD* mutant samples.

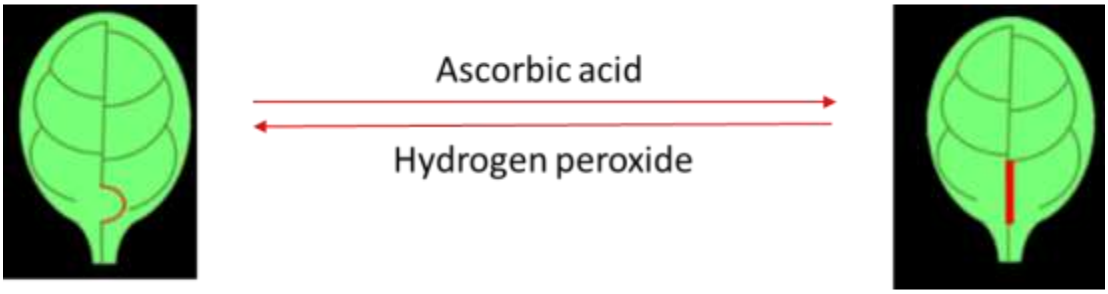
3.1.5 Level of ROS modulates path of reunion and polarised leaf vein network

Now, we asked that what would happen if we manipulate the ROS in mutants and in wild type. Thus, we performed the leaf vein regeneration assays using ascorbic acid and H_2O_2. Ascorbic acid (AsA) acts as a scavenging molecule that scavenges ROS which are produced during the various metabolic pathways (41) whereas H_2O_2 is a ROS which plays a vital role during various environmental conditions, which include both, the biotic as well as abiotic stresses (Dat et al. 2000). Various concentrations of AsA were used such as **50µm**, **75µm**, **100µm**. Similarly various concentrations of the H_2O_2 were also used such as **1mM**, **2mM**, **3mM** in leaf vein regeneration assay to see how does the level of ROS modulates the path of reunion. By adding quenching substance, we expect to get more of straight path of reunion as there would be less of

	Untreated control	**Ascorbic acid (ROS quencher)**	**Hydrogen peroxide (ROS source)**
Columbia-0 (wild type)	All the regenerated samples showed D-loop between physically disconnected vascular strands	Some of the regenerated samples showed **straight path of reunion** between physically disconnected vascular strands	No change as compared to untreated control (3mM hydrogen peroxide)
rbohD	D-loop and straight path of reunion was observed between physically disconnected vascular strands in **equal proportion** in regenerated samples	No change as compared to untreated control	Regenerated samples **showing D-loop were five times** the ones showing straight path of reunion between physically disconnected vascular strands **(2mM hydrogen peroxide)**
rbohF	Regenerated samples showing **D-loop were three times** the ones showing straight path of reunion between physically disconnected vascular strands	**No regeneration observed (75 µM ascorbic acid)**	No change as compared to untreated control

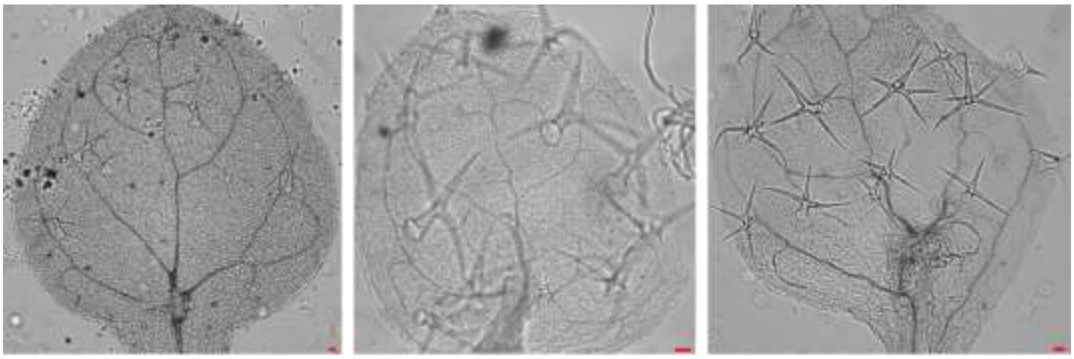
Table 4. Result of ROS modulation using quencher and ROS. A significant change was observed upon adding 75µm of Ascorbic acid (AsA) for the *rbohF* mutant. Similarly, when done for the hydrogen peroxide as well, an interesting observation was noted when 2mM was added locally to the *rbohD* mutant

ROS at the site of injury. Similarly, by adding ROS (H_2O_2) we expected more of D-loop formation because there would more ROS at the site of injury.



In untreated control samples of Columbia-0 almost all samples had D-loop as a way of regeneration but interestingly we got straight path of reunion in some sample wherein we have locally added the AsA to the samples which is as a quenching substance. But when done the same thing for *rbohF* there was **no regeneration** observed as oppose to D-loop which was three times the straight path of reunion in case of untreated control samples. This means that if we quench all the ROS molecule, we won't see regeneration because ROS is a signalling molecule. In case of *rbohD* as such no difference was observed with respect to the control.

When performed the same thing with H_2O_2 local application, now in case of *rbohD* we found 5 times more D-loop as oppose to when we had equal proportions of D-loop and straight path of reunion in case of untreated control *rbohD* samples. Now in case of *rbohF* and wild type no change was observed as compared to untreated control.



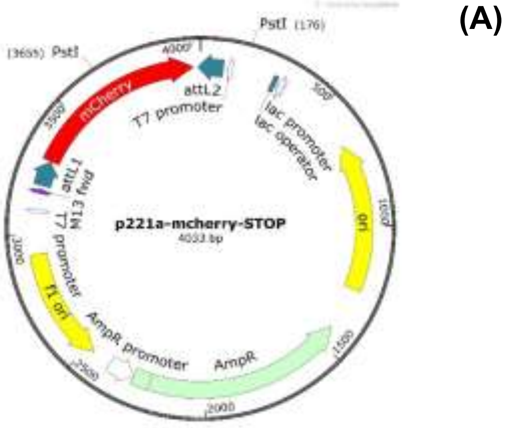
(A) Col-0 WT (Untreated) **(B) Col-0 WT (2mM H_2O_2)** **(C) *rbohF* (Untreated)**

Figure 18. Optimum level of ROS is necessary for polarised vein network. 18(A). Col-0 WT showing the normal straight leaf vein development when kept as an untreated control 18(B). Col-0 WT showing the wavy leaf vein development after adding the H_2O_2 locally 18(C). The Confocal imaging of the ROS mutant (*rbohF*) showing the wavy leaf vein development. Scale bars: 50µm

ROS is not only important in deciding the path of reunion between physically disconnected strand but also in proper formation of polarized leaf vein network. As shown in the figure 18 (A) we can see that there is normal straight vein development in case of Col-0 WT, but when added excess amount of ROS locally in the form of hydrogen peroxide (H_2O_2) with the concentration of 2mM, wavy vein development was observed in Col-0 WT {As shown in figure 18(B)}. Similarly, when observed the leaf vein development in case of the *rbohF* mutant, a wavy vein development was observed as *rbohF* is ROS deficient mutant {figure 18(C)}.

Section 3.2 Multisite Gateway Cloning

3.2.1 Confirmatory digestion of the Multisite gateway clones.



After the BP reaction of middle element of the construct pFRm43GW (spec, RFP) ZAT12::mCherry:NosT which is the reporter mCherry, a confirmatory digestion was using the *Pst*I restriction enzyme which had two cleavage sites on the plasmid. Upon virtual digestion of the same plasmid using benchling software, it showed two bands at 3508 bp and 583 bp. When ran the same plasmid on agarose gel after the BP reaction it showed the same banding pattern.

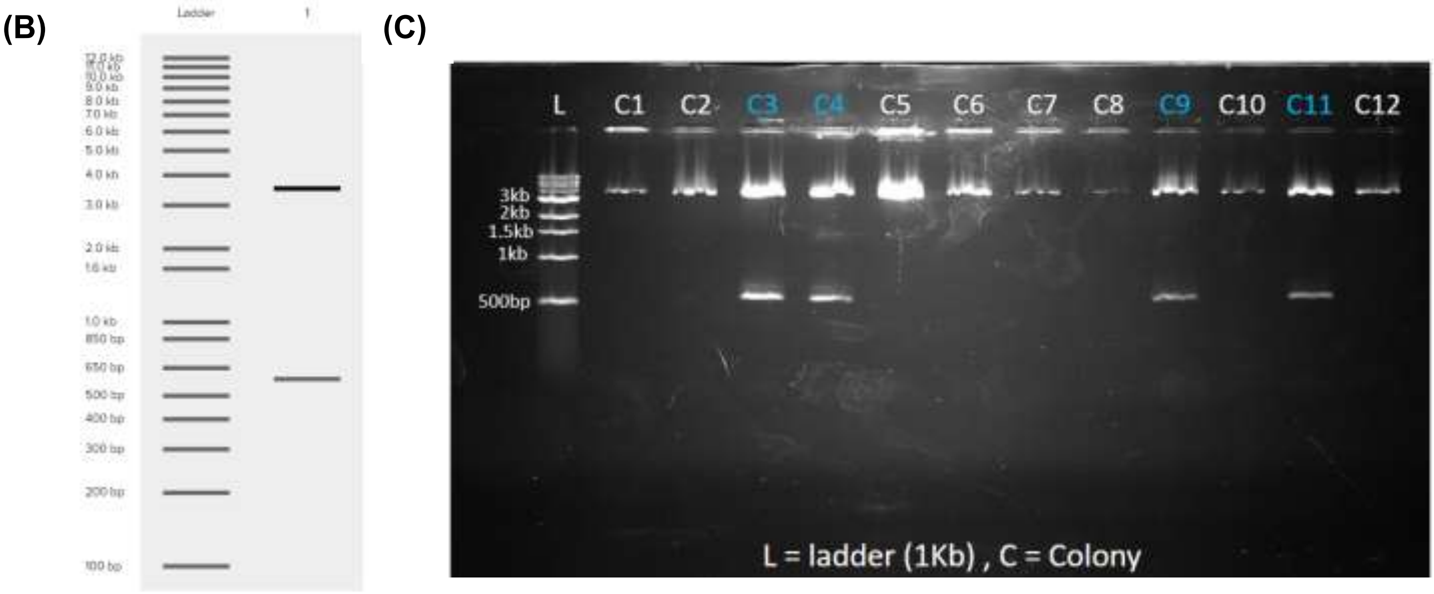
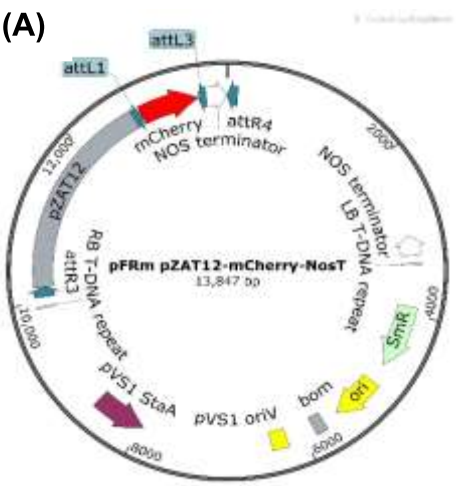


Figure 19: Confirmation of mCherry reporter fragment via gateway cloning. (A) p221a-mcherry-STOP cassette (B) Virtual digest of the plasmids along with Pst1 enzyme simulated using snap gene. (C) Out of the 12 colonies obtained after the DH5α transformation of mCherry BP reaction, the digested product was separated on 1% agarose gels and only 4 colonies were found positive.

When the LR reaction was carried at 25°C for 16 hours to combine all the three entry clones, in the presence of the destination vector who now holds up all the required components of the constructs namely:



1) p1R4-pZAT12 as a 5' entry clones holding the promoter of ZAT12.

2) p221a-mCherry as a middle entry clone holding the reporter mCherry.

3) 2R3 NosT as a 3' entry clone holding the terminator NosT.

A confirmatory digestion was carried out using the *Pst*I and *Eco*RV restriction enzyme which had two and one cleavage sites on the plasmid respectively. Upon virtual digestion of the same plasmid using benchling software, it showed three bands at **8668bp**, **2879bp** and **1560bp**. When ran the same plasmid **on agarose gel** after the LR reaction it showed the same **banding pattern.**

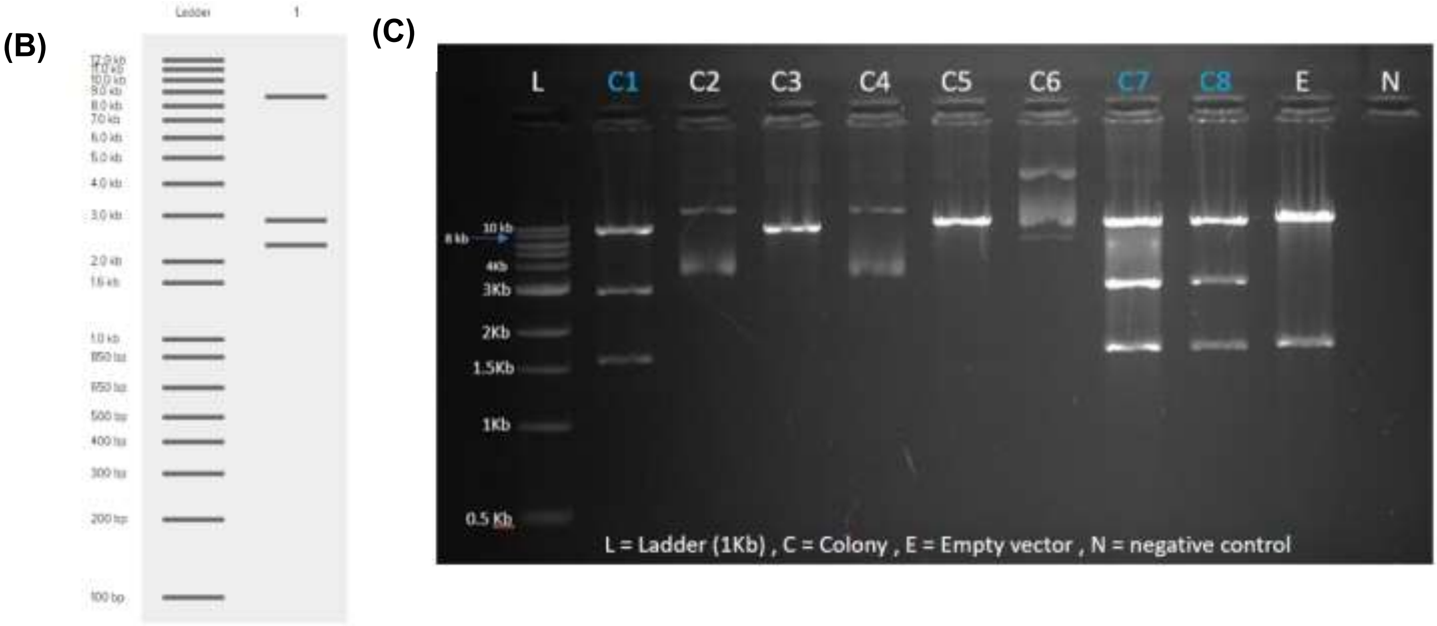


Figure 20: Confirmation of the LR reaction of the construct. (A) pFRm43GW (spec, RFP) ZAT12::mCherry:NosT cassette (B) Virtual digest of the plasmids along with Pst1 and Eco RI enzyme simulated using snap gene. (C) Out of the 8 colonies obtained after the DH5α transformation of LR reaction, the digested product was separated on 1% agarose gels and only 3 colonies were found positive.

3.2.2 Confirmatory PCR of *Agrobacterium tumefaciens* Transformation

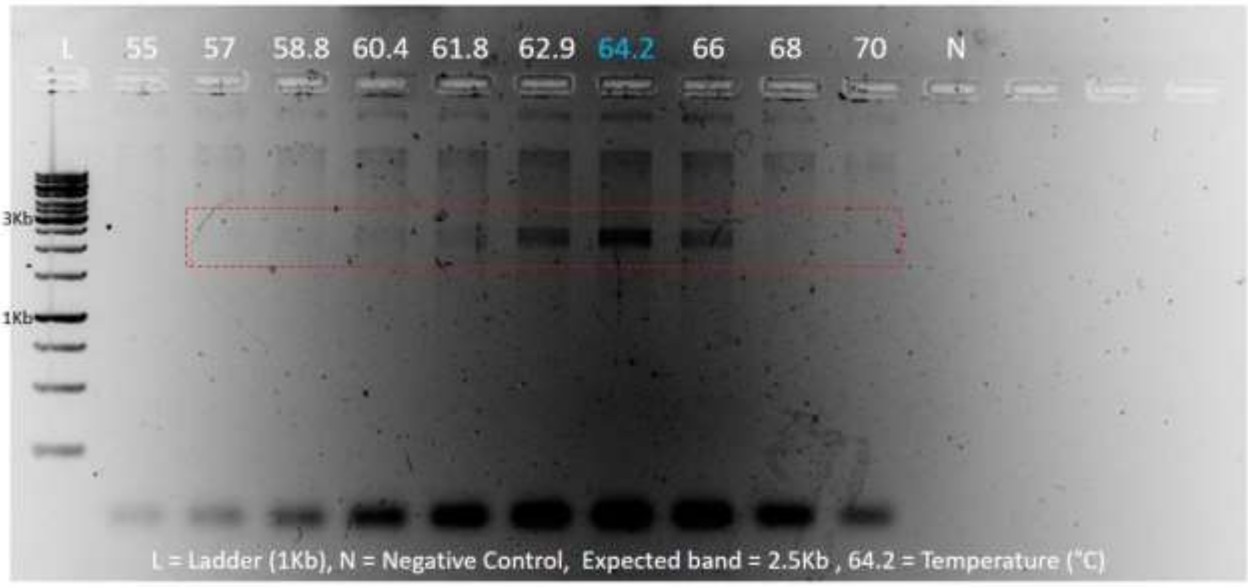
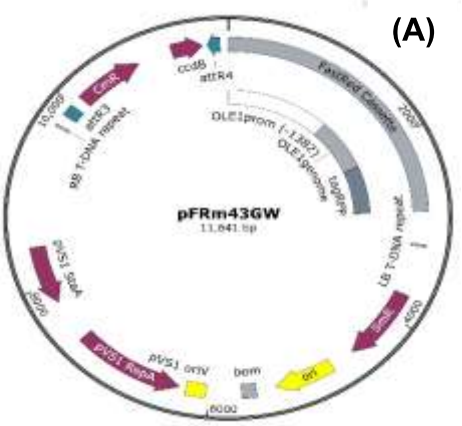


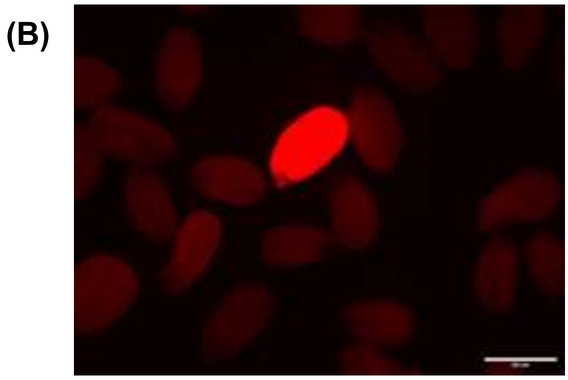
Figure 21: PCR analysis of confirmatory PCR products after the agrobacterium transformation. The Product were separated on 1% agarose gels after amplification with expecting band of 2.5Kb size. The band was observed most clearly at 64.2°C.

After the confirmation of the **pFRm43GW (spec, RFP) ZAT12::mCherry:NosT** construct, the C58 strain of agrobacterium was electroporated with the construct followed by a confirmational gradient PCR for screening the positive colonies containing our gene of plasmid. This PCR includes usage of temperature ranging from 55°C to 70°C and also the usage of primers which amplifies the promoter of ZAT12 as an indication of confirmation giving a band pattern at **2.5Kb** which is equivalent to size of the promoter used. Most promising band was seen at **64.2°C**.

3.2.3 Selection of Positive Transgenic Seeds with seed coat RFP

The destination vector which used was for the selection of positive transformants seeds from non-transformant seeds was **pFRm43GW** (Addgene ID 133748). Utilising the FAST (fluorescence-accumulating seed technology) Red cassette, this vector accumulates red fluorescence on the oil body membranes of developing *Arabidopsis thaliana* seeds, signifying the presence of a transformant. This process is based on the expression of OLE1 (OLEOSIN1) translational fusions, which is regulated by its native pOLE1 promoter.





Using a fluorescent stereomicroscope, the fluorescent seeds with RFP signal were selected in the first-generation following transformation (T1). When exposed such seeds to RFP channel under microscope showed the presence of bright red colour on their seed coat surface indicating the presence of our gene of interest in it. Such seeds were taken into consideration for further checking the actual working of the construct upon germination.

Figure 22: Transformed seed identification is facilitated by non-destructive screening markers. (A) T-DNA vector schematic illustration (pFRm43GW) containing the selection cassette called Fastred based on *pOLE::OLE1*-TagRFP expression unique to seeds. (B) Transgenic seed specimen with pFRm43GW which is identified using a fluorescent stereo zoom microscope.

3.2.4 Expression of the Bio-Transcriptional Reporter

ROS are the by-products of various enzymatic reactions in a cell. Most of the ROS production takes place in the Chloroplast, Mitochondria and peroxisome, as a part of the basal metabolic function. Based on the origins of reactive oxygen species (ROS), various cell types, and their tissue surroundings, ROS signalling participates in regular physiological functions or aids in the stress response.

When the ROS read out was taken with the help of bio-transcriptional reporter (**pFRm43GW ZAT12: mCherry: NosT**) in the Multiphoton microscope on the wild type uninjured five-day old germinated seedlings following observations were made: -

A. With the help of pFRm43GW ZAT12: mCherry: NosT bio-transcriptional reporter, it was observed that ROS production in non-stress condition was observed in region near to SAM (Dotted encircled region) and also in region around Midrib vein of the first rosette leaf (arrowhead) (Figure 8A).

B. When the ROS read was taken in the hypocotyl-root junction (Dotted encircled region) of the same sample, there was no ROS production seen in this area. (Figure 8B).

C. One difference which was observed in regions of apical meristems was that, ROS production was seen in region near SAM (Shoot apical meristem), but no ROS production was seen in RAM (root apical meristem, as was seen in the region of SAM. (Figure8C).

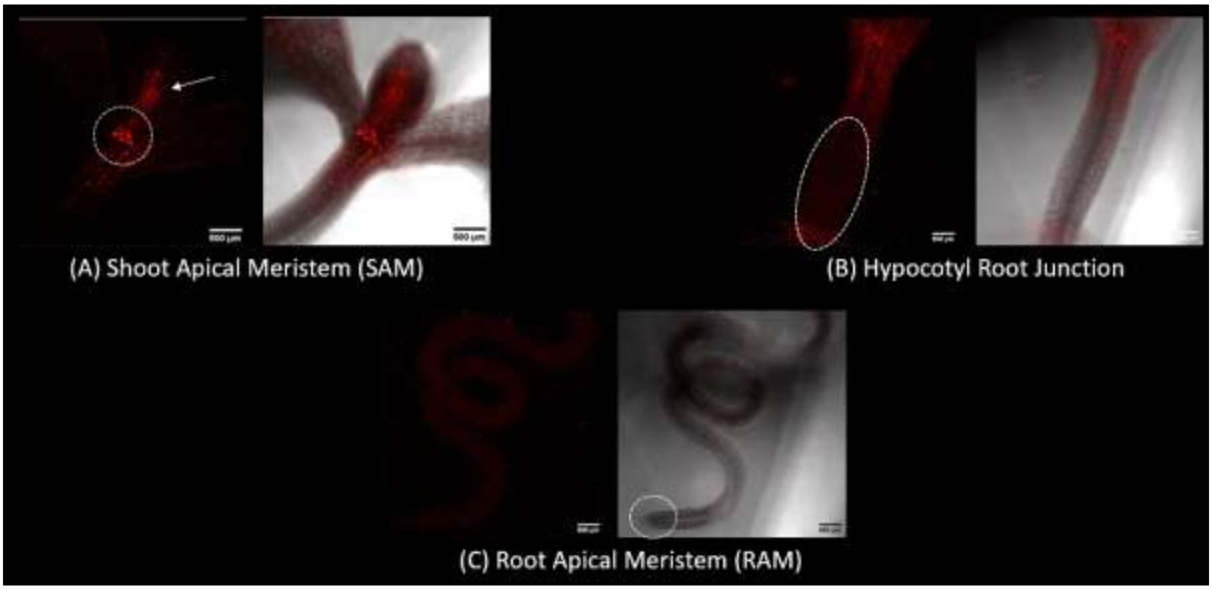


Figure 23: Imaging of ROS read with the help of bio-transcriptional reporter (pFRm43GW ZAT12::mCherry: NosT) on the wild type uninjured five-day old germinated seedlings. (8A) Shoot apical meristem (SAM) and midrib vein of first rosette leaf shows most ROS production because of effective contribution of major cellular activities. **(8B)** Hypocotyl root junction – No ROS production because of the absence of cell division and presence of cell elongation phase which doesn't produce enough ROS. **(8C)** Root Apical Meristem (RAM) – No ROS production signals were detected under multiphoton microscope. Scale bar 500 µm.

When the ROS read out was taken with the help of bio-transcriptional reporter (**pFRm43GW ZAT12: mCherry: NosT**) in the Multiphoton microscope on the ***Col; pg RBR-GFP*** background where RBR (Retinoblastoma-related) is G1 phase cell cycle marker, in an uninjured five-day old germinated seedlings following observations were made: -

- **A.** With the help of bio-transcriptional reporter, it was observed that ROS production in non-stress condition was observed in region near to SAM (Dotted encircled region) (Figure 25-A and A').

- **B.** When the ROS read out was taken in the region of root elongation (Dotted encircled region) of the same sample, there was seen a gradient of ROS production seen in this area where as RBR-GFP was seen majorly in area near RAM (root apical meristem). (Figure 25-B and B').

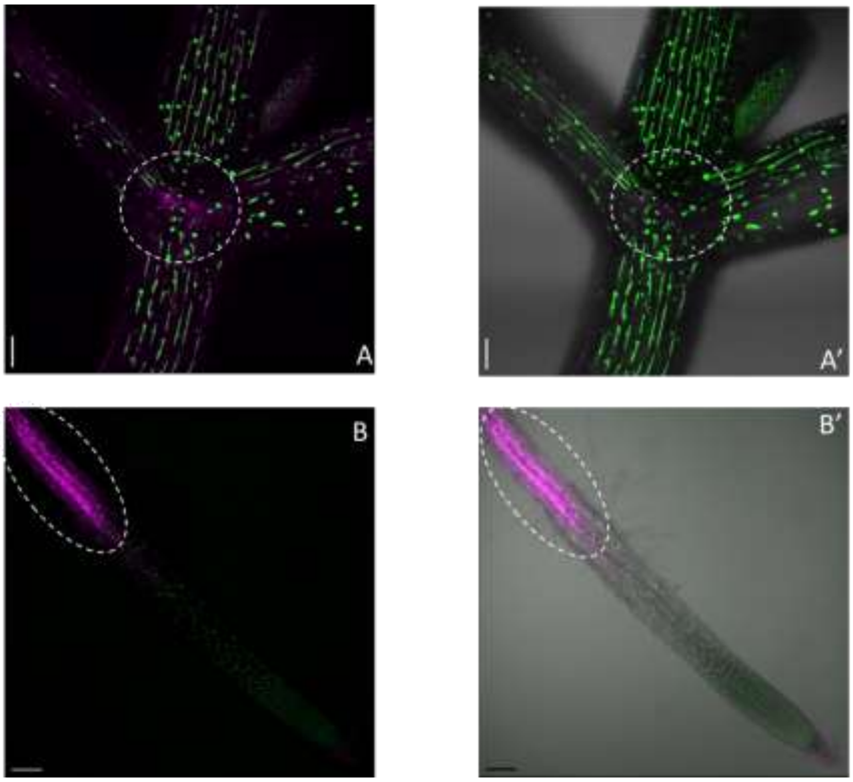


Figure 24: Imaging of ROS read out with the help of bio-transcriptional reporter (pFRm43GW ZAT12::mCherry: NosT) on the background *Col; pg RBR-GFP* in an uninjured five-day old germinated seedlings. (A and A') Near shoot apical meristem (SAM) region shows ROS production because of effective contribution of major cellular activities. **(B and B')** Region of elongation – gradient of ROS production was seen because of the cell elongation which is producing little ROS. Scale bar 500 µm.

Chapter 4 Discussion

To examine mechanically induced regeneration, one can see *de novo* root organogenesis (DNRR) from the cut end of a detached plant leaf and leaf vein injury as an example (Mathew and Prasad, 2021). Plants are vulnerable to several forms of harm and impairment, resulting from external or in vitro sources. Plants have developed a wide different types of regeneration responses (such as shoot or root regeneration, leaf vein regeneration, wound healing and vascular regeneration, root tip regeneration etc.) to help them survive these wounds and damage. The availability of nutrients and water is maintained in the mechanically injury-induced regeneration type, in which an injured or wounded organ is still attached to its parent.

Now in the case of DNRR from a separated organ, the leaf explant needs to live without any connection to the parent plant in order to survive (i.e., without nutrients and water availability from the parent). This is comparable to the natural process wherein a leaf breaks off from its parent plant and lands on the ground, where it forms roots and shoots before finally sprouting into a new plant. There have been new methods developed to imitate the natural DNRR response (Chen et al., 2014; Shanmukhan et al., 2021). These techniques allow us to examine the DNRR response and gain a better knowledge of the mechanism responsible for this kind of regeneration. Whereas when it comes to leaf vein regeneration, a different set of modules comes into play which is not applicable to normal leaf vein development. The injury is created to midrib vein then as response to that there is formation of either D-loop or straight pathway. This pathway is governed by the module which states that (Radhakrishnan et al., 2020) a coherent feedforward loop gets activated in response to vein injuries where members of PLT gene family activate the transcription of CUC2 gene. In order to activate the local auxin biosynthesis gene, YUC4, which in turn creates the ideal hormonal milieu for the reestablishment of polarised cell development and leaf vein regeneration, PLT and CUC2 work together in a coherent feedforward loop(Radhakrishnan et al. 2020).

These approaches to studying DNRR and LVR is well established in the plant model organisms called *Arabidopsis thaliana*. As a result, the objective of this study was to detect DNRR and LVR responses amongst the plant such as Columbia wild type and mutant species which were selected for the study. The results reveal that all of the mutant plants responded differently to the DNRR test when compared to the wild type of *Arabidopsis thaliana* (Figure 14). These findings complement what is found in case of leaf vein regeneration assays as well. Meaning as these mutants are of *rbohD* and *rbohF*, they lack major ROS producing metabolism as a result due to which the ROS which acts as a signalling molecule are absent in them thus their efficiency has went down as compared to wild type which accounts for nearly 32 percent and 15 percent for *rbohD* and *rbohF* respectively. Similarly, when it comes to affecting LVR, the level of ROS has become so low in *rbohD* that there is straight path of regeneration from the site of injury but also ROS had not vanished completely that it has resulted in formation of a D-loop. Thus, we are getting equal proportions of D-loop versus straight path of reunion. Now, when we the samples in case of rbohF we can say that the amount ROS was in such an optimum level where we could see the D-loop was three times the straight path of regeneration. Thus, if we want to conclude our observation in summary diagram (Figure 27) we can say that very less amount of ROS will not be helping in the process of regeneration because ROS acts as a signalling and in their absence the molecular mechanism of regeneration won't work properly. Similarly, if the amount of ROS is too high then due to the detrimental effect of the ROS, there would be NO regeneration. But depending upon the level of ROS we might end up getting different outcomes ranging from straight path of reunion to the D-loop formation.

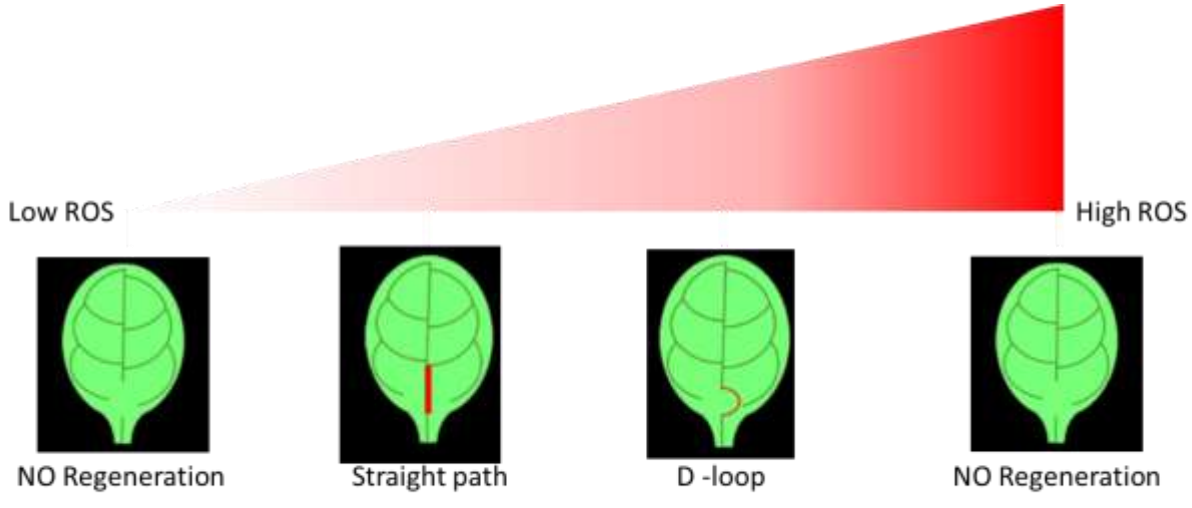


Figure 25: Summary model describing ROS in leaf vein regeneration. Near the both ends of the ROS levels we assume that no regeneration would take place. But an optimum level of ROS is required for leaf vein regeneration to take place.

The unique feature of the DNRR in *Arabidopsis* is its touch-dependent nature (i.e., WT-Root, WOT- Callus). To regenerate the root, a cut off leaf has to come into direct touch with any surfaces. (Shanmukhan *et al.*, 2021). To explain this phenomenon in more logical way a hypothesis is proposed which states that "Touch to the surface likely enable inhibitors of DNRR to leach out into the media, which would have otherwise accumulated at the cut end hindering DNRR"(Shanmukhan *et al.*, 2021). Meaning in case of non-touch the inhibiting factors are getting accumulated in the leaf as a result there is no formation of roots in case of non-touch but in case of touch (contact with surface) these inhibiting factors might be getting diffused out into the media as a result there is formation of root in this case. We are suspecting these inhibitors to be ROS (Reactive oxygen Species) which is making these difference in phenotypes in the regeneration process. Normally the ROS are produced at Basal metabolic rate (BMR) level as well where they act as signalling molecule. In this scenario these ROS are produced more in amount when any living has any sort of injury so as to acclimatize the stress situations. The same hypothesis is also applicable to LVR as well because it is the amount of ROS which decides the outcome, meaning in case of more ROS there is formation of D-loop and in case of less ROS there is linear pathway of regeneration. The next question is, what is the origin or molecular foundation of this form of *de novo* organogenesis? In the model plant organism called *Arabidopsis*, we looked at the ROS as a crucial factor for DNRR response. Taking the help of genetics and cloning a bio transcriptional reporter might be a possible way to answer some of the questions. With the use of ROS readout at the BMR (Basal metabolic rate) level, few observations were made. With the help of pFRm43GW ZAT12: mCherry: NosT bio-transcriptional reporter, it was observed that ROS production in non-stress condition was observed in region near to SAM and also in region around Midrib vein of the first rosette leaf Figure 8A. In the hypocotyl-root and at the Root apical meristem (RAM) junction there was no ROS production seen in this area. (Figure 8B).

Now, when we compare the data of DNRR, LVR and fluorescent staining we can to some extent speculate and say that there is a correlation observed between all of these findings. According to the hypothesis where we stated that ROS might be the one who is hindering formation of root in non-contact-oriented leaf and when we walk along the same line of thought and have started working with the mutants, a similar trend was observed. Meaning as mutant like *rbohD* had less of ROS compared to wildtype and *rbohF* therefore we were able to see more of DNRR efficiency only with respect to mutant and not wild type, which is being supported by the ROS quantification and a straight line of regeneration in LVR and on the other hand for *rbohF* there is more ROS comparatively with *rbohD* thus they might be hindering DNRR process thus giving us less regeneration efficiency and a regeneration in the shape of D-loop.

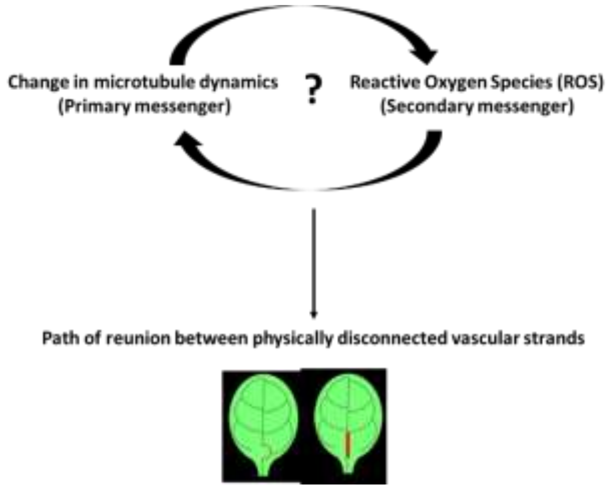
“ROS” guides regulatory module of regeneration?”

Reactive oxygen species, or ROS is crucial for several forms of regeneration, including LVR and DNRR. Given ROS is both adequate and required for DNRR and LVR, they may be the master regulators of these two variables' fate. This project's phenotypic data demonstrate the fact that these ROS as factors is essential for the mutant plant species and may function in the molecular network to produce responses like *de novo* root and leaf vein regeneration. If all of the above information is right, there is a possibility that ROS (particularly H_2O_2) directs regulatory module of regeneration in plant species just because as compared to other ROS produced, it has highest lifetime which might end up affecting regeneration to a greater extent.

Future aspects

Knowing the fundamentals of DNRR and LVR research is crucial to comprehending the process of plant regeneration. Still, a lot of questions remain unanswered: Why does the cutting part of the leaf have to come into contact with its environment in order for new roots to grow? In what form must ROS signalling be occurring at the cut end? What sets apart the linear path of regeneration from the D-loop formation? Thus, to study genetics of leaf vein regeneration, double and triple mutants would be made by artificial crossing with the *clasp* (plus end binding protein in microtubule) which includes: -

- *rbohD* x *rbohF*
- *rbohD* x *clasp*
- *rbohF* x *clasp*
- *rbohF* x *rbohD* x *clasp*



As microtubule is primary messenger for sensing the stress condition, by coupling it with the ROS, which is a secondary messenger for sensing the stress, we would be able to figure out how does the interplay between these two messengers would affect the various regeneration assays including leaf vein regeneration assays.

Furthermore, to investigate the cell biology of the leaf vein regeneration various crossing and transformations have been thought to elucidate such mechanism which includes: -

- *rbohD*/ATHB8-RFP
- *rbohD*/PXY-YFP
- *rbohD*/WOX4-YFP
- *rbohD*/CYCB1-GFP
- *rbohD*/SOK3-YFP
- *rbohD*/KA1-mCitrine
- *rbohD*/DR5-3xVENUS
- *rbohD*/PIN1-GFP
- *rbohD*/TUB6-GFP
- *rbohD*/CLASP-YFP
- *rbohF*/ATHB8-RFP
- *rbohF*/PXY-YFP
- *rbohF*/WOX4-YFP
- *rbohF*/CYCB1-GFP
- *rbohF*/SOK3-YFP
- *rbohF*/KA1-mCitrine
- *rbohF*/DR5-3xVENUS
- *rbohF*/PIN1-GFP
- *rbohF*/TUB6-GFP
- *rbohF*/CLASP-YFP

- ATHBB8 is precursor of procambium cells.
- Pxy (phloem intercalated xylem) is required for polar divisions of procambial cells.
- WOX (stem cell marker) helps in mesophyll to procambium dedifferentiation.
- DR5 – auxin sensor (Auxin helps in differentiation of procambial cell).
- PIN 1 - auxin polar transporter.
- SOK 3 – cell polarity marker (cell of vein r polar thus forming intense network of vein).
- Cyclin B1 - cell cycle marker (M phase marker).
- TUB6 – Helps in Tubulin formation.

In order to address these issues, more investigation is needed into the cellular and molecular sources of the LVR and DNRR in the *Arabidopsis thaliana* model plant. Moreover, examining regeneration in several plant species enables us to comprehend regeneration from an evolutionary standpoint.

Limitations of the study

While screening the Wild type and mutant plant species for the DNRR, there were some other regenerative responses, such as callus formation from the cut end in With Touch. Because this study focused on the DNRR, such responses were not taken into investigation further

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