# Characterisation of intragenic cis regulatory modules in transcription during de novo shoot regeneration

A 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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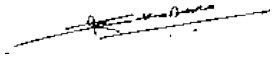
INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

# Certificate

This is to certify that this dissertation Characterisation of intragenic regulatory modules in transcription during de novo shoot 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 Ghodke Shruti Prakash at Indian Institute of Science Education and Research, Pune 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.

# Declaration

I hereby declare that the matter embodied in the report entitled Characterisation of intragenic regulatory modules in transcription during de novo shoot regeneration are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Kalika Prasad and the same has not been submitted elsewhere for any other degree

Ghodke Shruti Prakash

Date: April 10, 2023

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# Abstract

Plants along with all other organisms play a vital role in the ecosystem. However unlike birds or animals, they cannot move. To save themselves from their predators, plants evolved various strategies. Various strategies also evolved so as to sustain the injuries and maintain their species on the planet.

Plants possess remarkable property of regeneration. The process of regeneration, like all other life processes is carried out with fine tuning of various transcription factors. Plants regenerate in various different ways.

Mechanisms underlying these processes have been studied for quiet some time now. Recent studies from the lab revealed important information about CUC2 a shoot promoting factor and XTH9 a cell wall regulating enzyme. It revealed the regulatory mechanisms underlying shoot formation. The main motive of this project was to explore this regulation in greater depth.

# Acknowledgments

I would like to thank Dr. Kalika Prasad for me giving me an opportunity to pursue my master's thesis under his guidance. I would also like to thank Srijan Das for his guidance and valuable insights throughout the project. I would like to thank Akansha, Anju, Mabel, Vijina and all other members from the MGPB lab for their help and guidance.

I would like to express my gratitude towards my family for believing in me. I would like to thank Yadhusankar, Saket , and my fellow batchmates for their help.

# Contributions

| Contributor name | Contributor role |
|---|---|
| Dr.Kalika Prasad | Conceptualization Ideas |
| Srijan Das | Methodology |
| - | Software |
| - | Validation |
| Srijan Das, Shruti Ghodke | Formal analysis |
| Shruti Ghodke | Investigation |
| - | Resources |
| - | Data Curation |
| Shruti Ghodke | Writing - original draft preparation |
| Shruti Ghodke | Writing - review and editing |
| - | Visualization |
| Dr. Kalika Prasad | Supervision |
| - | Project administration |
| - | Funding acquisition |

# Chapter 1 Introduction

Plants harbour tremendous regenerative potential. Entire plant can be generated from an explant. There are two modes of regeneration, tissue culture mediated regen`eration and mechanical injury induced regeneration. In tissue culture induced regeneration, entire organism is produced from an explant. De novo organogenesis is the term for this event. De novo organogenesis involves de novo root regeneration and de novo shoot regeneration. De novo organogenesis can be direct as well as indirect. Direct de novo organogenesis does not involve a callus. Callus is a mass of undifferentiated cells. Shoots emerge from lateral root primordia (LRP). Indirect de novo organogenesis involves the development of a callus from the explant and then further to a root or a shoot.

## Arabidopsis as a model organism

Arabidopsis Thaliana, is one of the most widely studied model organism. It is a plant from the Brassicaceae family and is native to Europe, Asia and Africa. It has a fast life cycle (produces seeds in 6-8 weeks), grows upto a height of 30  cm, thus can be grown conveniently inside a growth chamber, are some of the characteristics that make Arabidopsis Thaliana a good model organism. Arabidopsis Thaliana genome has been sequenced. The genome consists of 20,000 protein coding genes. Arabidopsis genome has five chromosomes (Arabidopsis Genome Initiative 2000; Cheng et al.2017). Thus a multicellular organism with five chromosomes, makes Arabidopsis a feasible model organism. It has a relatively simple genome and a short life cycle, making it an ideal plant for research purposes. The plant has a rosette-like structure with leaves emerging from a central point, and it produces small white flowers. The formation of callus involves the activation of root stem cell regulators like ABERRANT LATERAL ROOT FORMATION4 (ALF4), which plays a crucial role in the initiation of cell division and dedifferentiation of cells into a pluripotent state.

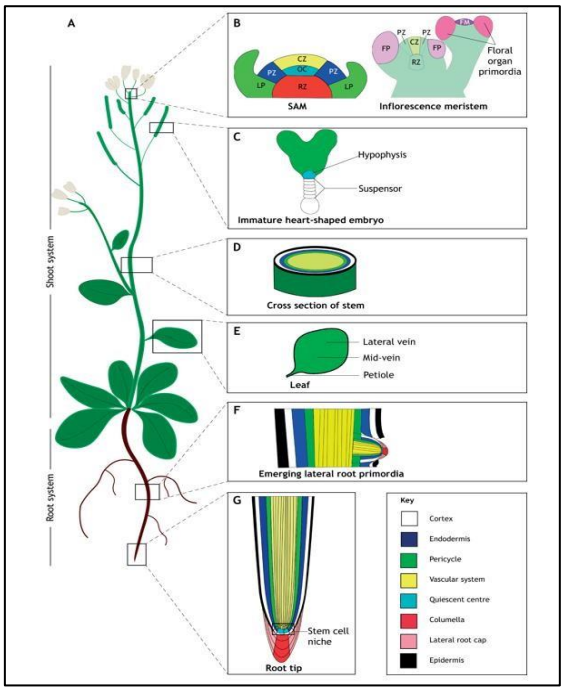


*Figure 1 : Schematic representation of Arabidopsis (B) The stalk apical meristem (SAM), which normally produces a leaf at its flank, transforms into an inflorescence meristem and produces a flower at its flank as the plant moves from the vegetative phase to the reproductive phase. The stem cell niche is made up of the organising core (OC) and the central zone (CZ) within the SAM. (SCN). A juvenile heart-shaped embryo is shown in the inset (C) of the silique. (fruit). (D) The inset shows a cross-section of the inflorescence stalk with various tissues visible. The leaf's mid vein, lateral vein, and stem are shown in the inset in (E). (F) The inset shows the development of lateral roots. The root tip is shown inset in (G) with various tissues visible. Within the root apical meristem, the SCN is made up of the quiescent centre and the cells that encircle it. *Image taken from Radhakrishnan et al., 2018*

However, the callus cells must develop pluripotency in order to finish the process of shoot or root regeneration. The PLETHORA (PLT) genes, specifically PLT7, PLT5, and PLT3, are essential to this two-step molecular process. PLT genes play a role in the positioning of lateral

organs during the growth of roots and shoots. In order to prepare the callus cells to produce shoot progenitors during regeneration, the PLT genes first activate the regulators of root stem cells.

The regeneration of a full shoot is accomplished in the second step by the PLT genes activating proteins that promote the growth of new shoots, such as CUP-SHAPED COTYLEDON 2 (CUC2). No matter the developmental origin of the explants, this two-step procedure is constant.

The PLT genes are expressed in a spatially and temporally regulated manner during regeneration. They are initially expressed in the periphery of the callus, where they activate the expression of root stem cell regulators. As the callus cells become pluripotent, the PLT genes are expressed throughout the callus, promoting shoot regeneration.

Overall, the molecular mechanism of callus formation and regeneration involves the activation of root stem cell regulators by PLT genes, followed by the activation of shoot-promoting factors to regenerate a complete shoot. This two-step mechanism is very important for the successful regeneration of plants from tissue culture.

The plant hormones auxin and cytokinin cause the process of de novo shoot regeneration, which is mediated by a number of transcription factors (TFs) particular to plants. High auxin concentrations promote callus development, which can further differentiate into different cell types.

A callus is comprised of a few progenitor cells that can start the process of developing into a shoot meristem, which in turn gives rise to a full shoot system. Specific transcription factors (TFs) that control gene expression and cell fate determination are activated during this process.

De novo shoot regeneration, is regulated by a complex network of signaling pathways and gene regulatory mechanisms. This process involves the transformation of pluripotent callus cells into specialized progenitor cells, which can then differentiate into shoot meristem cells and eventually into a complete shoot system.

In conclusion, de novo shoot regeneration is an intricate and strictly controlled procedure. The auxin and cytokinin plant hormones act as the trigger for the process, which involves the activation of transcription factors that control cell differentiation and fate determination.

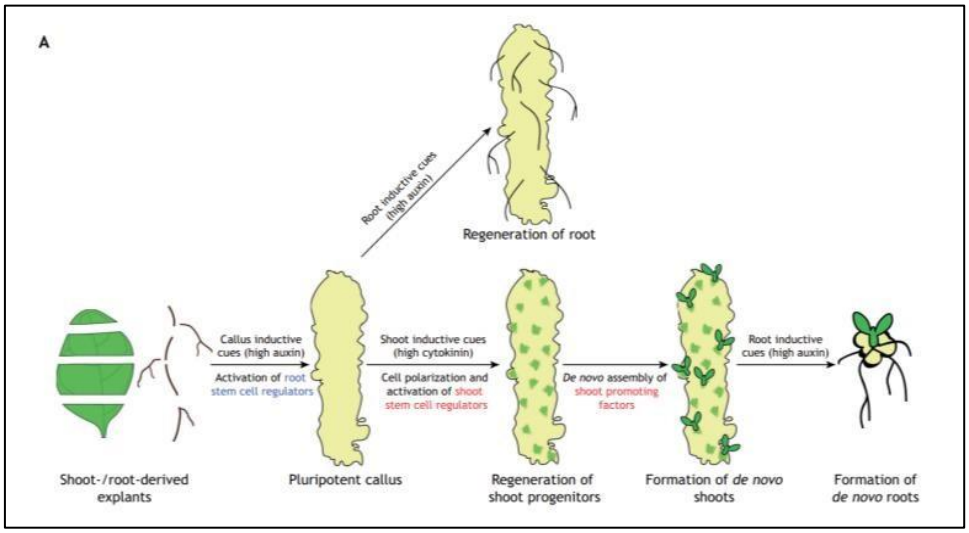


*Figure 2: Illustration of de novo shoot and root regeneration. *Image taken from Varapparambath et al., 2022*

A comparative transcriptome analysis was done so as to identify the regulators responsible for promoting a specific pattern of PIN1 protein localization observed in regenerating progenitor cells. The study compared three different genetic constructs:

| | |
|---|---|
| *pPLT7::cPLT1-vYFP;plt3,plt5-2,plt7* | Callus consisting of progenitor cells however shoot meristem not formed |
| *pPLT7:cPLT1-vYFP,p35S::CUC2-3AT;plt3,plt5-2,plt7(a double reconstitution callus)* | Progenitor cells were capable of progressing into functional shoot meristem |
| *p35S::CUC2:GR;WT* | Upon DEX induction, higher number of progenitors seen. |

2592 genes were elevated in DEX-treated CUC2-GR samples compared to mock treatment. There were 218 genes in common between the elevated genes in DR and the upregulated genes in DEX-treated CUC2-GR.

To know more about these 218 genes and their functions, a gene ontology analysis was done. It was discovered that these genes were more highly expressed in metabolite interconversion enzymes, which included contained hydrolases. One type of class of enzymes, called hydrolases, uses the addition of water molecules to catalyse the dissolution of chemical bonds. The XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE (XTH) gene family was enriched for the members XTH7, XTH9, XTH24, and XTH32.

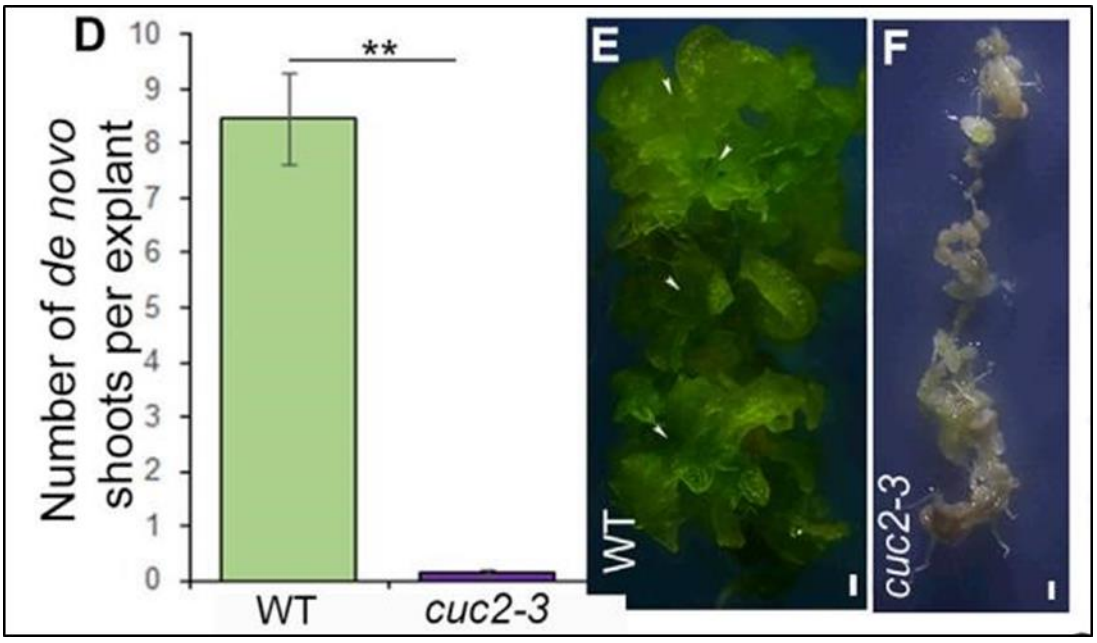


*Figure 3: D, Graph illustrating decrease in the number of de novo shoots as compared to wild type E,F callus with shoots grown *Image taken from Varapparambath et al., 2022*

# CUC2-XTH9 regulatory module

XTH9 was downregulated in cuc2-3 calli, shoot growth from the callus had significantly decreased as compared to wild type callus.

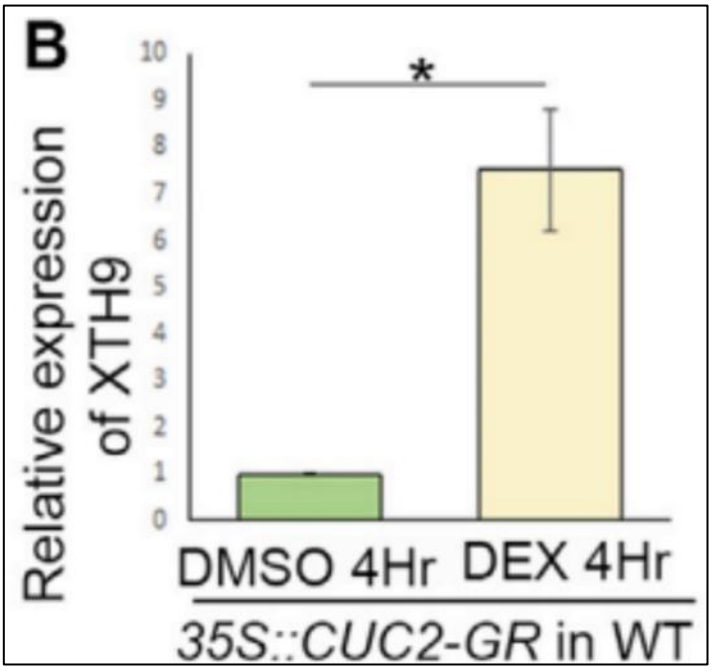


*Figure 4: Image showing upregulation of XTH9 expression in p35S::CUC2-GR when induced grown *Image taken from Varapparambath et al., 2022*

Using pCUC2::CUC2:vYFP;WT, ChIP-seq analysis during progenitor onset was done . It was discovered that XTH9 has three important CUC2 binding sites, including one strong motif on exon 4 and two weaker binding motifs on exons 1 and 3.

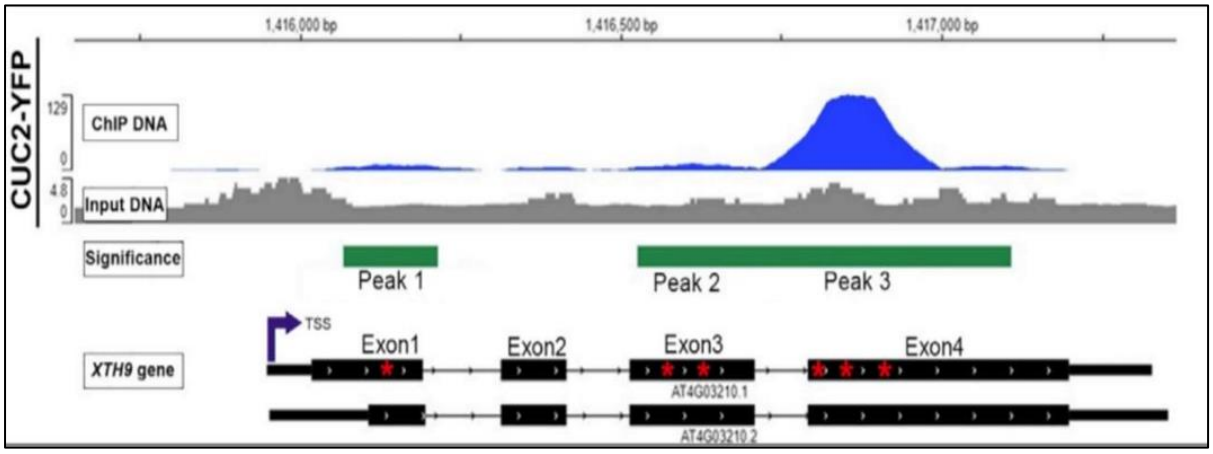


*Figure 5: Binding motifs of CUC2 on XTH9, grown *Image taken from Varapparambath et al., 2022*

All these evidences point to the fact that CUC2 directly activates the transcription of XTH9 . ( Varapparambath et al.,2022). There have been many other such evidences of transcription factors binding to exonic part of genes.( Ritter et al.,2012).

Analysis of ChIP seq data revealed that CUC2 binds to exonic region of one more gene throughout Arabidopsis genome. MEE38 , Maternal Effect Embryo Arrest, is the gene on whose sixth exon CUC2 binds.

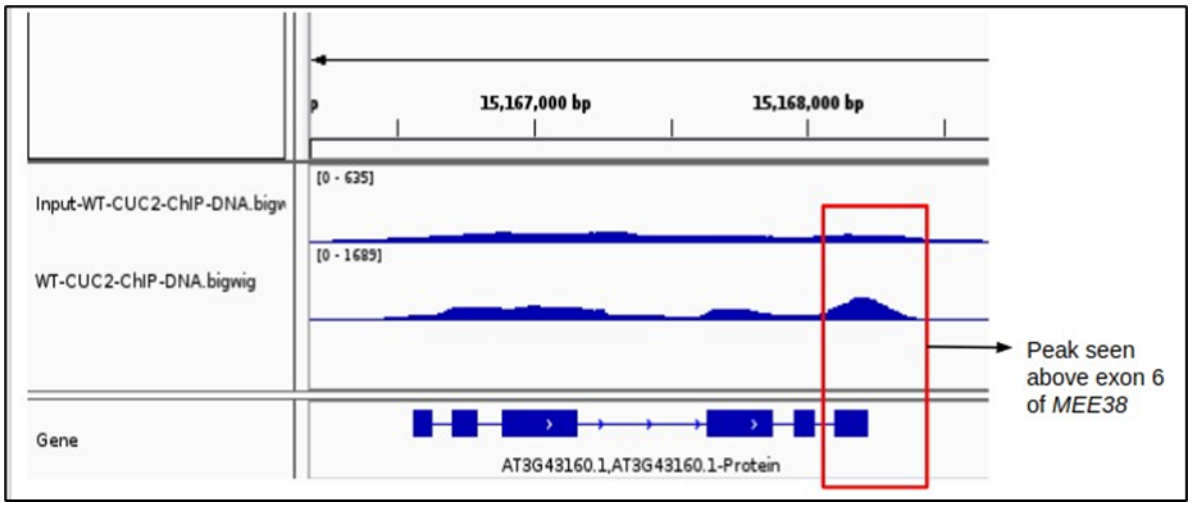


*Figure 6: Binding peak of CUC2* seen above exon6 of *MEE38* (Image taken from Integrated Genome Viewer)

# Gene regulation

Gene regulation is the process by which cells control the expression of genes. The regulation of gene expression is critical for cells to function normally and to respond to changes in their environment. RNA processing, translation, post-translational modification of proteins, transcription, and other mechanisms can all be used to control gene expression. Cis and trans regulatory modules are two types of DNA sequences involved in the regulation of gene expression. Cis regulatory modules (CRMs) are stretches of DNA sequence that are located near the gene they regulate. Trans regulatory modules (TRMs), on the other hand, are proteins that bind to DNA and regulate gene expression. These proteins can be transcription factors or other regulatory proteins that are produced by genes that are located elsewhere in the genome. Cis regulatory modules consists of regulatory regions of DNA, such as promoters, enhancers, and silencers. These regulatory regions contain binding sites for specific transcription factors, which can either activate or repress transcription of the gene. Promoters are DNA sequences located upstream of a gene that are involved in initiating the transcription of the gene into RNA. They serve as binding sites for RNA polymerase, the enzyme responsible for synthesizing RNA from DNA, and other regulatory proteins that control the rate of transcription.

Promoters consist of a core promoter, which includes the transcription start site and the binding sites for RNA polymerase, and regulatory elements, which are located further upstream and can enhance or repress the activity of the core promoter. Silencers, also known as negative regulatory elements, are DNA sequences that act to repress or decrease the level of gene expression. They are typically located upstream or downstream of a gene, and can function by binding to transcription factors or other regulatory proteins that inhibit the activity of the promoter. Enhancers are DNA sequences that can increase the level of gene expression when bound by transcription factors or other regulatory proteins. They are typically located upstream, downstream, or within the gene they control, but can function over long distances to influence gene expression. Enhancers play a vital role in regulating gene expression by providing a mechanism for precise control of the level and timing of gene expression. They can be activated in response to a wide range of stimuli, such as hormones, environmental cues, or developmental signals, and can work with other regulatory elements, including promoters and silencers, to regulate gene expression.

As mentioned above, CUC2 positively regulates XTH9 expression. CUC2 has binding sites on exon4 of XTH9 and exon6 of MEE38. The main objective of this thesis was to explore this

regulation further and characterize what these exonic sequences function as. It was hypothesized, based on all the previous findings, that exon4 of XTH9 can act as an enhancer. Exon6 of MEE38 also had binding motifs for CUC2. To characterize a sequence as an enhancer, various methods can be used. Here we decided to do a gene regulatory assay which involved making a construct of three gene fragments. First fragment was the exonic sequence, followed by a minimal promoter, followed by a reporter gene. If the exonic sequence is an enhancer, it will drive the minimal promoter, and as a result expression of the reporter gene will be seen, and it can be concluded that the exonic sequence was an enhancer.( Weber et al.,2016).

# Chapter 2 **Materials and Methods**

## Cloning

1) **Multisite gateway cloning:**
   It is a cloning method based on site specific recombination. Multiple DNA fragments can be integrated into a vector in specific orientation and sequence through this method. It involves two reactions, BP reaction and LR reaction, which involve usage of enzymes BPclonase and LRclonase respectively.
2) **Designing primers:**
   We used Integrated Genome Viewer for analysing the ChIP seq files. Concerned DNA sequences were then obtained from TAIR. Primers were designed using Snapgene. For XTH9 exon4, primers were designed for a fragment of 301bp flanked by attB sites. For MEE38_exon6, primers were designed for a fragment of 412bp flanked by attB sites. For, minimal p35S primers were designed for a fragment of 47bp flanked by attB sites. All three sets of primers were designed using snapgene. Primer sequences are mentioned below:

| | |
|---|---|
| *MEE38*_ex6_1R4_FP | GGGGACAACTTTGTATAGAAAAGTTGTTGGTTGGCGGTATATAACTTTTGC |
| *MEE38*_ex6_1R4_RP | GGGGACTGCTTTTTTGTACAAACTTGTAAAGCACCTTAATCGAAGATTC |
| *XTH9_ex4*_1R4_FP | GGGGACAACTTTGTATAGAAAAGTTGTTATTCATGGTAGACGAAACACC |
| *XTH9_ex4*_1R4_RP | GGGGACTGCTTTTTTGTACAAACTTGTATGATTCTGATGAAGGCTAAGC |
| Min35S_221_FP | GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGCAAGACCCTTCCTCTATATAAG |
| Min35S_221_RP | GGGGACCACTTTGTACAAGAAAGCTGGGTTTTCTCTCCAAATGAAATGAACTTC |

*Table 1: Primer sequences used for gateway cloning.*

3) **Amplifying the exonic sequences:**
   XTH9exon4 and MEE38exon6 were amplified from Arabidopsis Thaliana genomic DNA isolated using CTAB method, using above mentioned primers. PCR reagents as follows:

   - Genomic DNA
   - Prime star GXL polymerase
   - Prime star GXL polymerase 10Xbuffer
   - dNTP
   - Sequence specific primers
   - Nuclease free water(NFW)
   - 200ul PCR tubes
   - BIORAD Thermocycler

   Gradient PCRs were set up for each of the three sequences, and an annealing temperature was selected so as to get the correct amplification. At the selected annealing temperatures, bulk PCRs were set up . Amplicon was isolated from the reaction mixture through gel elution using MN gel

   NucleoSpin Gel and PCR Clean-up, Mini kit for gel extraction and PCR clean up.
   Insert (i.e. PCR product flanked by att sites) and backbone plasmids is taken in 1:3 ratio of amount with BP clonase enzyme, and incubated at 25 deg celsius for 12-16 hours. Reaction is terminated by adding Proteinase K and incubating reaction mixture at 37 deg Celsius for 10 minutes.

Constructs were then transformed to DH5α E.coli bacterium through heat shock mediated transformation. Bacterial plates were then kept at 37 degree Celsius for 12 hours. Plasmids were isolated and further screened by restriction digestion .

Positive plasmids for constructs, 1R4PGEMT:min35S, 221z:XTH9_exon4, 221z:MEE38_exon6, were used for further cloning.

These entry vectors were then used to set up a LR reaction with R3R4 vector using LR clonase enzyme. Reaction mixture consisting of specific entry vectors, destination vector R3R4, LR clonase enzyme, was then incubated at 25 deg Celsius for 12-16 hours , reaction was terminated by adding Proteinase K and was incubated at 37 deg Celsius for 10 minutes. Construct was then transformed to DH5α strain of E.coli through heat shock. The transformed construct was then plated on LA plates containing suitable antibiotic (here Rifampicin and Kanamycin was used) . Plate was incubated at 37deg Celsius for 12-16 hours. Further plasmids were isolated from the colonies and screened by restriction digestion ( EcoR1 and HindIII were used for-XTH9_exon4:min35S:Vyfp and Pst1 for MEE38_exon6:min35S:vYFP)

Positive plasmids were then transformed into C58 strain of Agrobacterium

Tumifaciens through electroporation. Transformed construct was then plated on LA plates with Rifampicin and Kanamycin. Plates were incubated at 29 deg Celsius for 48 hours. A colony was picked from each of the plates and inoculated. Wild type Arabidopsis Thaliana plants (Col) were then transformed using floral dip method.

**4) Plant transformation:**

Arabidopsis Thaliana plants were grown in pots. Plants were grown so as to let them grow many immature flower clusters. A day before transformation, all the siliques of plants were removed.

A primary culture of C58 strain of Agrobacterium was prepared for two constructs(pmin35S:XTH9_exon4::vYFP and pmin35S:MEE38_exon6::Vyfp) in 5ml LB with 5µl Rifampicin(50µg/ml) and 5µl Kanamycin(20µg/ml) . Culture was inoculated at 29C for 12-24 hours. 2ml of this primary culture was then added to 200ml of LB with with 20µl Rifampicin(50µg/ml) and 20µl Kanamycin(20µg/ml) and this secondary culture was then inoculated at 29C ° for 12-14 hours.

Above two cultures were then pelletized by centrifugation at 6000rpm for 15 minutes at room temperature and resuspended in 100ml of 5% (weight/volume) sucrose solution.

0.04% silwet L-77bwas added to above solution, and this solution was transferred to an empty tip box.

Using a 1ml pipette the sucrose, culture and silwet mixture as made above, was transferred onto each and every cluster of floral buds. The solution was then drained after 3-5 seconds. A soapy film of liquid was visible on the buds. The plants were then covered with a black plastic bag to maintain constant humidity and the plants were shifted to growth chamber. Post 16-24 hours, the black plastic bag was removed, and a transparent plastic bag was placed surrounding the plants. Plants were allowed to grow for a month and were watered regularly . Siliques started turning brown and the seeds were ready to be collected post a month after transformation. Seeds were then collected in 50ml falcons.

5) **Screening of transgenic seeds:**

Collected seeds from both the constructs were sterilized .Seed sterilization was done inside Laminar Air Flow to ensure sterile conditions. 50ml of 70% ethanol was added to both the falcons and invert mixed for 4-5 minutes. A spin of 2 minutes at 6000rpm was given for 2 minutes so that all the seeds settle down. Ethanol was decanted and 50ml of 20% sodium hypochlorite was added to the falcon, and invert mixed for 3 minutes. A short spin was given at 6000rpm and sodium hypochlorite was added.

Further the seeds were washed with autoclaved RO water 7 times ( 5 minutes per wash). A brief spin was given after each wash.

Seeds were then stored in 50 ml of autoclaved RO water at 4C for cold ˚treatment for 3-4 days.

Seeds were plated on ½ strength MS( Murashige and Skoog)

Ampicillin+Hygromycin plates and placed vertically in plant growth chamber. Transformed seeds have Hygromycin resistance, as a result they will germinate on a Hygromycin infused media. Seeds that germinated on these plates were then transferred to ½ strength MS( Murashige and Skoog) plates post 7 days.

Post 7-10 days, seedlings were potted. Plants are kept in the growth chamber. Stable transgenics will be grown so as to perform further experiments

Seeds from the transformed plants were collected, sterilized and plated on MS media with Hygromycin antibiotic. Further seedling with antibiotic resistance were selected and potted. Few more stable generations of the transgenics need to be generated for performing further assays.

6) **Media preparation:**

- ½ MS (Murashige and Skoog) media:
  0.5375gm MS salt and 2.5gm sucrose was dissolved in 245ml of autoclaved MilliQ water in a reagent bottle . pH of this solution was then adjusted to 5.7 using 1N KOH, and volume was then made up to 250ml. 1.8gm of plant agar was added and the media was autoclaved. Autoclaved media was then cooled slightly, and 250ul of Ampicillin was added (stock concentration of Ampicillin is 100mg/ml) was added to 250ml media. After swirling the bottle,

  250ml media was poured into 4 circular glass plates ( roughly 62.5ml/plate) . Once cooled, the media sets, and the plates were packed with Cling film and stored in cold storage for further use.

# Chapter 3 Results

Both the constructs were confirmed using restriction digestion

- *XTH9_exon4::min35S:YFP plasmid digestion ( 11064bp)*

  Expected banding pattern, 6594bp, 1945bp, 1721bp, 493bp, 311bp

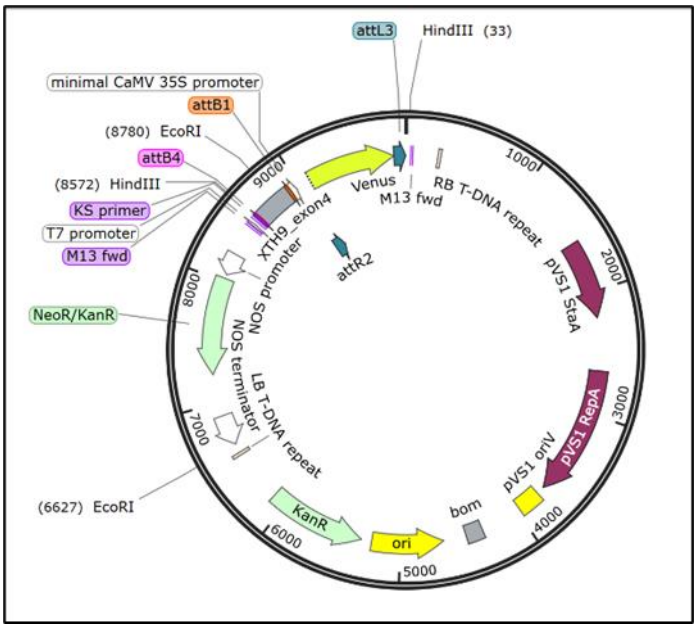


*Figure 7: Plasmid map of for XTH9_exon4::min35S:YFP plasmid*

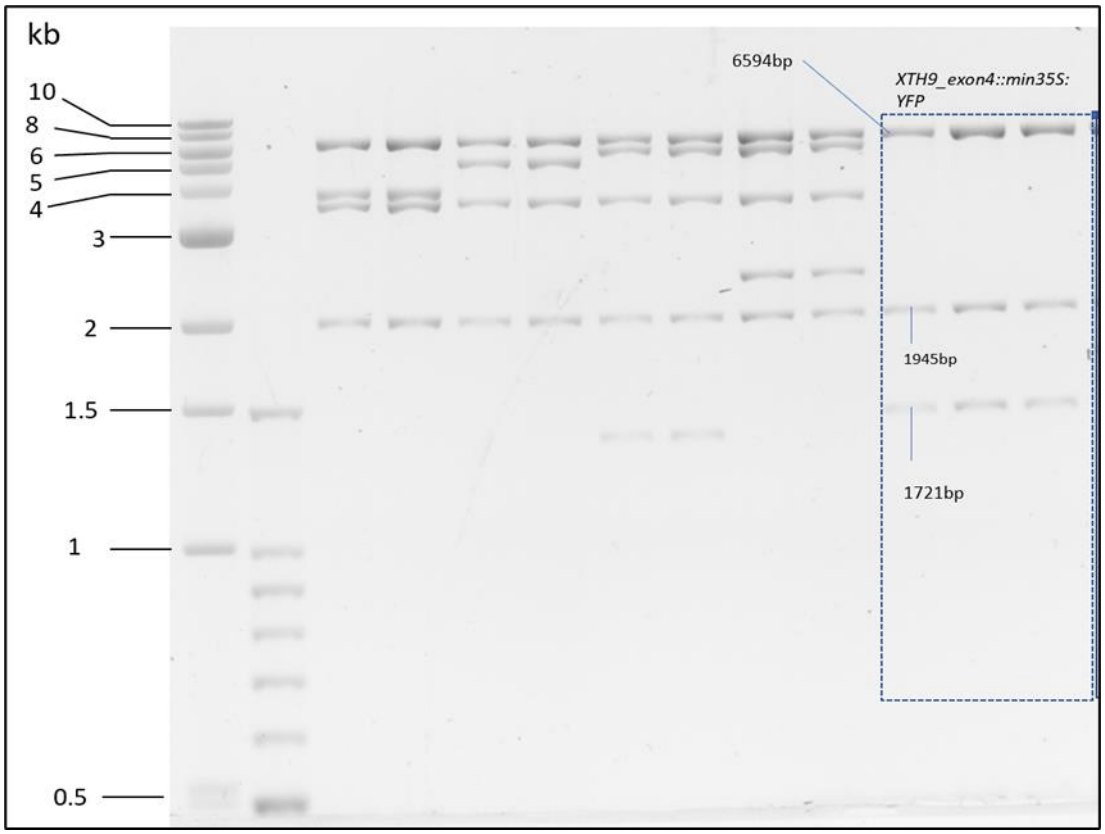


*Figure 8: Gel image of restriction digestion for XTH9_exon4::min35S:YFP plasmids* .Blue rectangle denotes the positive plasmids. First well has 1kb DNA ladder

Mee38 plasmid digestion using EcoR1Hf and BamH1Hf

Expected banding pattern, 6624bp, 2301bp, 1722bp, 446bp, 17bp

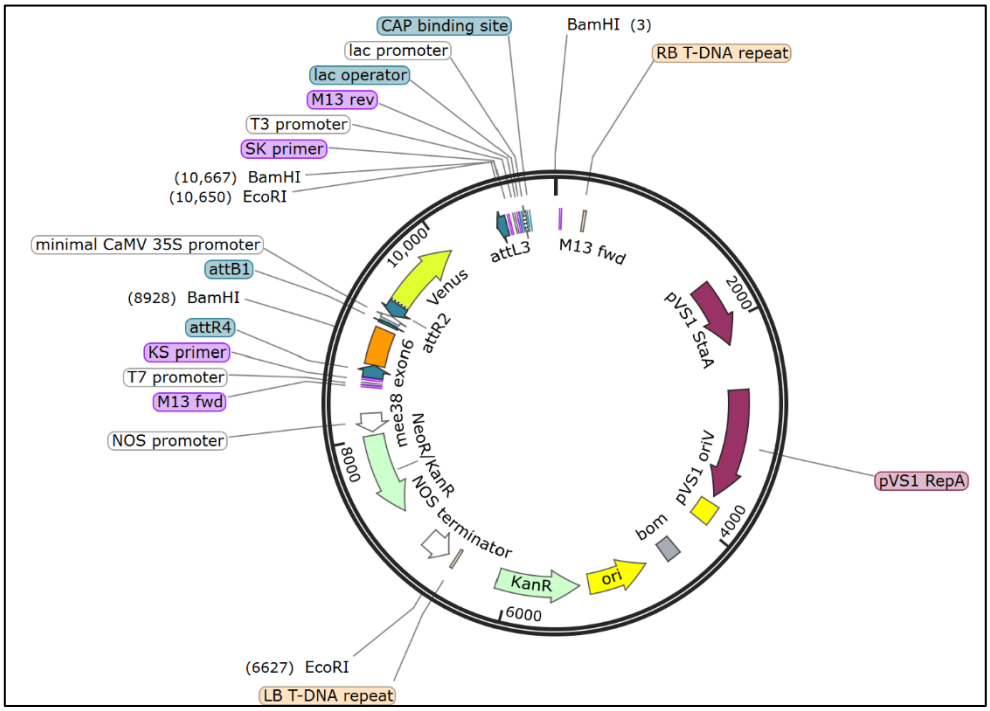


*Figure 9: Plasmid map of MEE38_exon6::min35S:YFP*

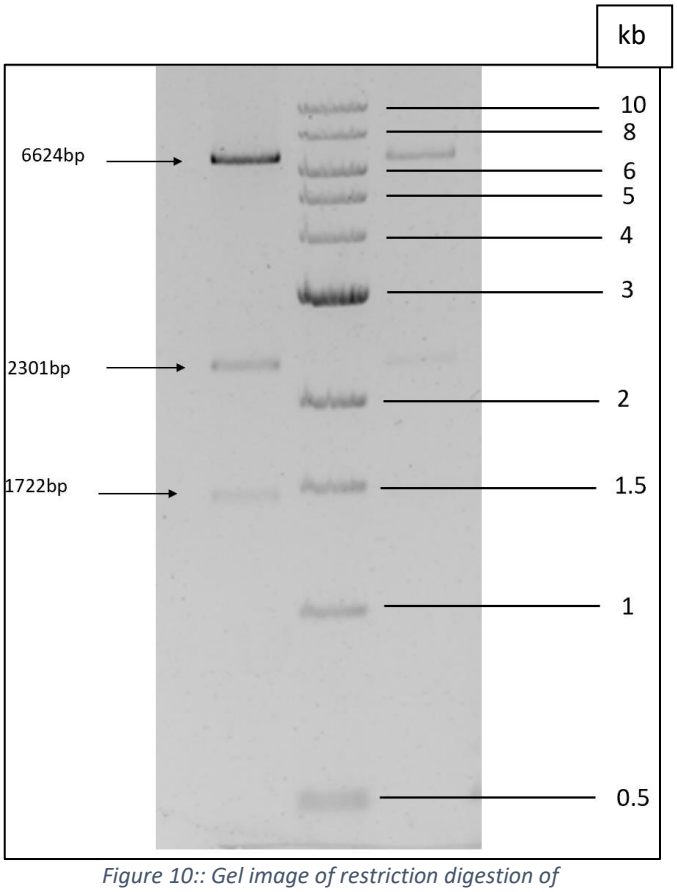


*Figure 10:: Gel image of restriction digestion of MEE38_exon6::min35S:YFP plasmids*

Post transformation, seeds were collected from the transformed plants and screened using antibiotic resistance. Seeds that germinated were then screened using microscopy. Explant of the seedling was used. Here root of the seedling was used. For *MEE38_exon8::min35S:vYFP,*some signal was seen at the root tips. As seen in the image below, signal was seen at the root tip in some explants of the transgenics. *YFP* expression was seen, this could be because of various reasons. Due to the complex web of regulatory networks that exist in various organisms, the exact factors behind this expression cannot be claimed at this point.

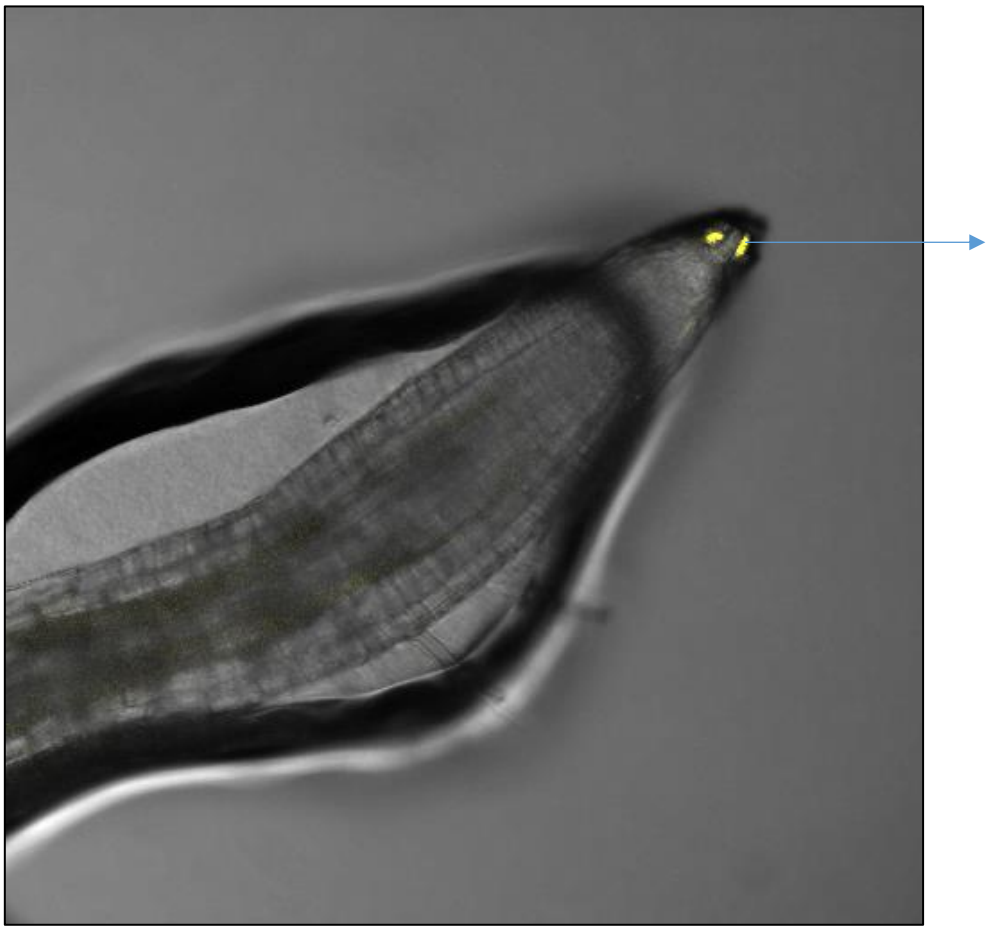

*Figure 11: YFP expression seen in a MEE38_exon6::min35S:vYFPexplant*

# Chapter 4 Discussion

CUC2 is a shoot promoting factor (Kareem et al.,). To characterize its regulatory elements, experiments need to be done at the time of shoot formation. Therefore, to check how these two constructs, i.e XTH9exon4:min35S::Vyfp and MEE38exon6:min35S::Vyfp function, callus needs to be screened while on the onset of producing progenitors. Callus is the right tissue to be screened. For that, two more generations of the transgenic plants need to be grown, so as to obtain a stable line. Therefore to characterize these two exonic sequences, further experiments will be done in future using seeds collected from future generation of these transgenics.

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