Inducible CRISPR/CAS9 genome editing to modulate autophagy during *de novo* organ regeneration.

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled "**Inducible CRISPR/CAS9 genome editing to modulate autophagy during *de novo* organ 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 Yadhusankar S at Indian Institute of Science Education and Research under the supervision of Dr. Kalika Prasad, Associate Professor, Department of Biology, during the academic year 2022-2023.

Dr. Kalika Prasad

Committee:

Dr. Kalika Prasad

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This thesis is dedicated to my sister.

Declaration

I hereby declare that the matter embodied in the report entitled "**Inducible CRISPR/CAS9 genome editing to modulate autophagy during *de novo* organ regeneration**" are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Kalika Prasad and the same has not been submitted elsewhere for any other degree

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Abstract

Regeneration is a natural process which gets activated upon injury and helps in reorganisation of the lost body parts in such a way that it regains its full functionality. At the cellular level, one of many intriguing questions to ask is how the cell fixes its integrity during an extensive damage condition and how it forms an organ *de novo*. Autophagy is identified as one of the cell survival mechanisms that help in maintaining homeostasis in damaged cells by the elimination of damaged organelles. However, little is known about the pathways that could link autophagy and regeneration in the plant kingdom. Therefore, this study on creating knockouts of autophagy genes with Inducible CRISPR/Cas9 Genome Editing would potentially throw light on the molecular and genetic regulation of regeneration via autophagy in wound-induced *de novo* organogenesis.

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Contributions

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Akansha Ganguly	Validation
-	Formal analysis
Yadhusankar S	Investigation
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Yadhusankar S	Data Curation
Yadhusankar S	Writing - original draft preparation
Yadhusankar S	Writing - review and editing
-	Visualization
Kalika Prasad	Supervision
Kalika Prasad	Project administration
-	Funding acquisition

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

Chapter 1: INTRODUCTION: REGENERATION

1.1 Regeneration in Organisms

Humanity has always been fascinated by regeneration, which is evidenced in old writings. Ancient Greek mythology mentions the beast known as the hydra, which can sprout two heads when one is severed, as well as Prometheus' liver regenerating as an endless punishment by Zeus. Advancements in science and technology have helped us to understand the basis of regeneration at cellular, genetic, and molecular levels. Regeneration can be considered conserved but is very limited in higher organisms in the animal kingdom (Poss, 2010). The commonly used animal model systems to study regeneration include planaria, hydra, axolotl, tadpole, etc. and it can be noted that the regenerative property reduces with an increase in the complexity of the organism (Fig. 1.1).

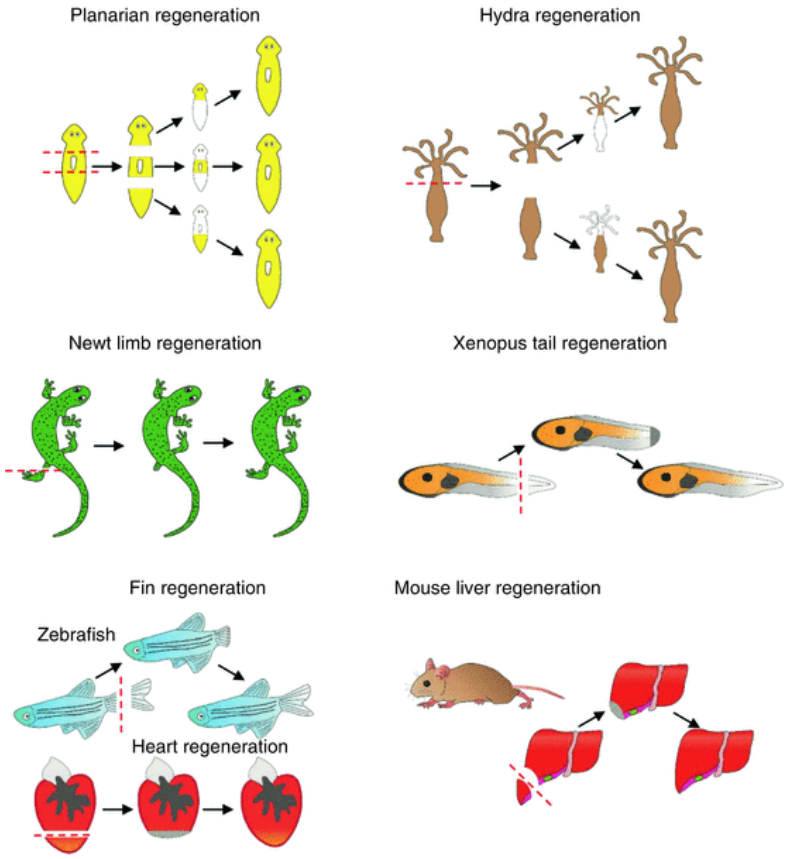


Fig. 1.1: Regeneration in Animal Kingdom. (Image Courtesy: Fu et al., 2018)

1.2 Regeneration in Plants

Plants are sessile and have limited motility. They are mostly confined to the site of germination and have evolved various strategies to overcome the environmental adversities they have to face throughout their lifetime. Nevertheless, plants possess immense developmental plasticity. Unlike other species on this planet, they continue to grow quickly with the help of meristematic regions at the root and shoot tip. It has been shown that some plant cells can essentially dedifferentiate or transdifferentiate into stem cells to give rise to progenitors that could result in *de novo* organogenesis (Chatfield *et al.*, 2013; Liu *et al.*, 2014; Kareem *et al.*, 2016) This can happen in two ways: Wound-induced or hormone-induced which can be termed as direct or indirect regeneration, respectively (Fig. 1.2). In hormone-induced *de novo* regeneration, a higher auxin content in the growth media can give rise to a callus, which under subsequent hormonal cues can form a shoot or a root. In wound-induced regeneration, the detached or injured organ repairs itself via pathways that are little known to the scientific world (Chen *et al.*, 2016; Iwase *et al.*, 2017; Ikeuchi *et al.*, 2022). How the organ fixes the cell integrity at the wound site despite the extensive cell damage is a question that needs to be addressed in detail.

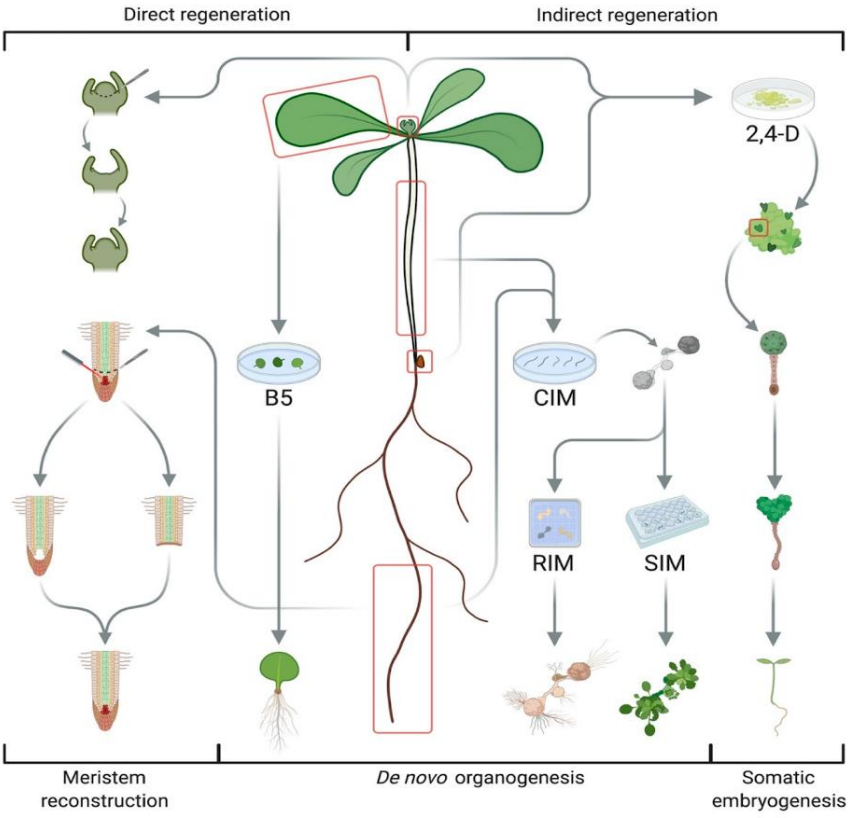


Fig. 1.2: Types of regeneration in plants. (Image Courtesy: Lardon and Geelen, 2020)

1.3 *De novo* root regeneration (DNRR) from leaf petiole

For millennia, people have been observing the *de novo* root formation from a plant's detached leaf, as well as exploiting this occurrence as a means of propagating various plant species. In *Kalanchoe* leaves, regeneration of the entire plant could be observed which serves as a means for the propagation of the species, occurring in nature. Recent studies on this organogenesis have revealed that the mechanism by which the root form from the leaf is touch-dependent (Fig. 1.3). The formation of the root occurs without any exogenous hormonal or nutrient supply. Interestingly, when the detached end of the leaf is placed without touching a media, it forms a callus, independent of the nature of the medium (Shanmukhan *et al.*, 2021). The regeneration-regulating transcription factors called *PLETHORA (PLTs)* along with *WOX11*, *LBD16*, and *ERF115* are found to be upregulated in the detached leaves during DNRR (Xu *et al.*, 2006; Shanmukhan *et al.*, 2021; Liu *et al.*, 2022). Other genetic as well as molecular factors that could potentially play a role in this process remain unknown and needs to be studied in detail.

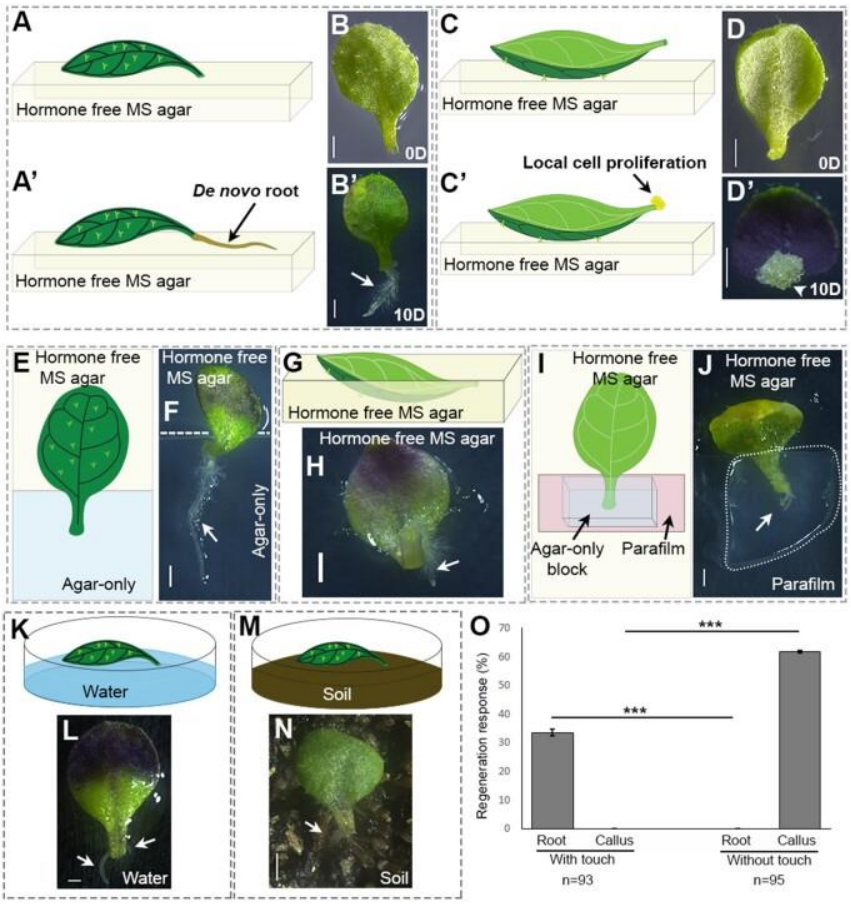


Fig. 1.3: Assay for touch-dependent de novo root regeneration (Image Courtesy: Shanmukhan et al., 2021)

1.4 Autophagy and Regeneration

In regenerative biology, it is crucial to understand how nearby cells ensure their survival when a wound is produced. Some of the proposed mechanisms by which cells maintain their integrity upon extensive damage is autophagy. Autophagy in plants is classified into three broad categories: microautophagy, macroautophagy, and megaautophagy. Among these, macroautophagy is widely studied and known to be regulated via the formation of autophagosomes. A plethora of gene regulatory networks has been identified that have been shown to play a significant part in the modulation of autophagy. Among these are the *Autophagy related genes (ATGs)*, which is conserved across Eukaryotes. There are around 42 genes that are reported under the *ATG* superfamily (Furukawa *et al.*, 2019; Cao *et al.*, 2021). Among them, *ATG8* and its isoforms are identified to be crucial in the formation and transport of autophagosomes to the vacuole where they are degraded. Nine isoforms of *ATG8* have been reported so far. In the touch-dependent root regeneration assay, *ATG8* gene expression is downregulated in PLT deficient background in the *de novo* root formation from the detached *Arabidopsis* leaves (Akansha Ganguly, unpublished data). For further studies, RNAi silencing of *ATG8* isoforms is in progress but gene editing techniques like CRISPR/Cas9, Cre/Lox-based clonal deletion, and transcription activator-like effector nuclease (TALEN)-based genome editing and Zinc finger Nuclease, etc. are reported to be more efficient in creating mutant lines (Fig. 1.4). Among these, CRIPSR/Cas9 is the most reliable method with less cost and we decided to proceed with it to create knockouts for isoforms of *ATG8*.

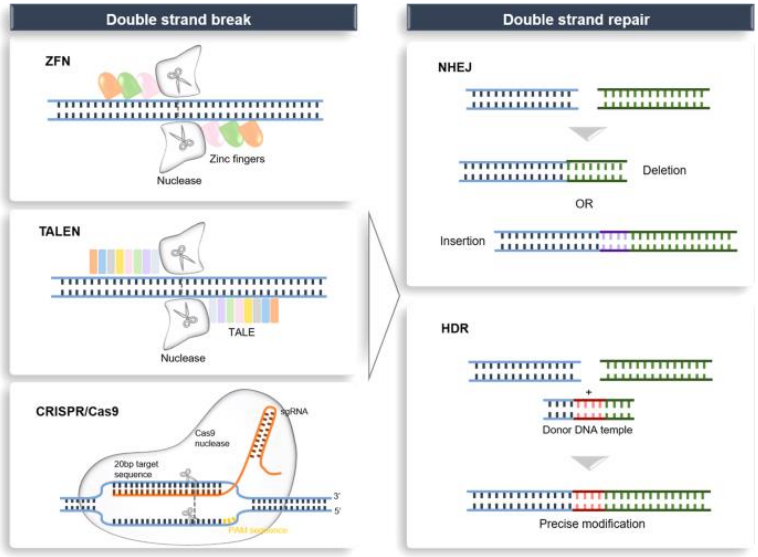


Fig. 1.4: Commonly used Genome editing systems. (Image Courtesy:(Li et al., 2020)

Chapter 2: INDUCIBLE CRISPR/CAS9 GENOME EDITING

2.1 CRIPSR/Cas9 in Bacterial System

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), a crucial component of the bacterial defence system (Babu *et al.*, 2011; Loureiro and da Silva, 2019), uses DNA sequences from bacteriophages that have already infected the bacteria to recognize and cleave similar DNA sequences upon secondary infection. The CRISPR part of the system involves using RNA molecules to guide a protein called Cas9 to a specific location in the DNA sequence. Once Cas9 is at the right spot, it can cut the DNA, which can then be repaired or altered by the cell's own repair mechanisms. Molecular biologists use this system to make accurate alterations to specific DNA sequences, such as adding or deleting genes or correcting mutations.

2.2 Plant-optimized CRISPR/Cas9

Plant-optimized CRISPR/Cas9 is a genome editing tool that has been specifically designed to target and modify plant genomes(Cong *et al.*, 2013; Ma *et al.*, 2015). The CRISPR/Cas9 system consists of two main components: the Cas9 enzyme, which acts as molecular scissors that cleave DNA, and the guide RNA (gRNA) molecule, which directs Cas9 to a specific location in the genome (Fig. 2.1). To optimize the CRISPR/Cas9 system in plant system, researchers have made several modifications to the Cas9 enzyme and the gRNA molecule. For example, they have modified the Cas9 enzyme to make it smaller, which allows it to be delivered more easily into plant cells. They have also made changes to the gRNA molecule to improve its specificity, which minimises the likelihood of effects that are off-target. The use of plant-optimized CRISPR/Cas9 has several advantages over other genome editing techniques. First, it is highly efficient, meaning that it can achieve targeted modifications in a high proportion of cells. Second, it is highly specific, meaning that it can target specific genes without affecting other genes in the genome. Finally, it is relatively easy to use and could be applied to a great range species of plant kingdom.

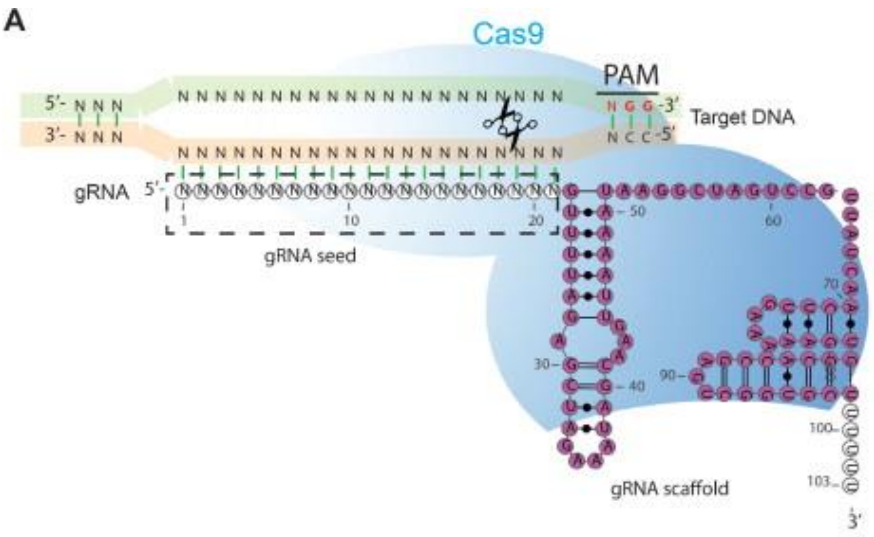


Fig. 2.1: Short guide-RNA mediated genome editing in plants using CRISPR/Cas9 system (Image Courtesy: Xie and Yang, 2013)

2.3 Inducible genome editing (IGE) system

Inducible genome editing by CRISPR/Cas9 is a very powerful tool that allows the precise control of spatial and temporal regulation of gene editing in living cells (Wang *et al.*, 2020). This technology has significant potential for advancing our understanding of gene function. Inducible CRISPR/Cas9 systems work in such a way that the editing process can be triggered or controlled by external stimuli such as light, chemicals, or temperature changes. The inducible system allows for the temporal and spatial control of the CRISPR/Cas9 gene editing machinery, which is critical for studying gene function and disease modelling.

There are several ways to create an inducible CRISPR/Cas9 system, but one common approach is to use an inducible promoter to control the expression of the Cas9 gene. The inducible promoter is a DNA sequence that is activated by a specific stimulus, such as a chemical compound or light. When the inducer is added or the light is turned on, the promoter is activated and the Cas9 protein is produced (Fig. 2.2(B)). The Cas9 protein can then be guided to the target DNA sequence using a guide RNA (gRNA) that is specific to the gene of interest.

2.4 IGE to create knockout for *ATG8B, ATG8C, ATG8F* & *ATG8H*

CRISPR/Cas9 has recently shown robust activity in plant systems using plant codon-optimized CAS9 gene. The PCR-based procedure helped to generate multiple sgRNA expression cassettes with a higher mutation rate compared to that of the existing gene-editing tools, and these mutations were expressed in progenies. Cas9-mediated genome editing can also be used in accordance with an XVE-based cell-type-specific inducible system to knock out genes in desired cell type at desired time point (Fig. 2.2(A)). I developed the knockout for isoforms of *ATG8* namely *ATG8B, ATG8C, ATG8F* and *ATG8H.* For the sake of simplicity, I am describing the methods and experiments for one of the isoforms, *ATG8F*.

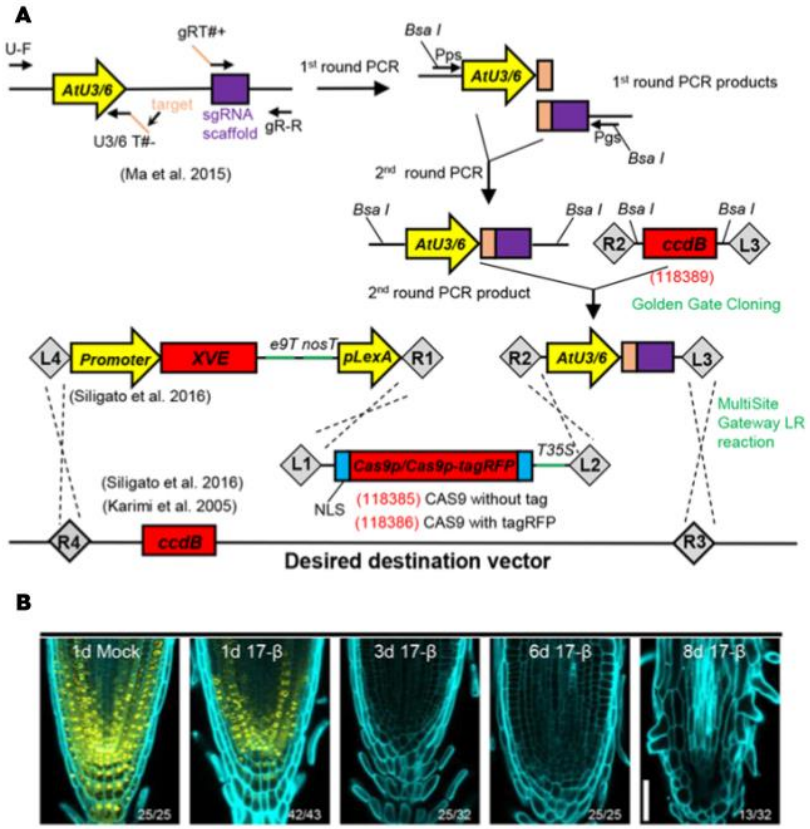


Fig. 2.2: Inducible Genome Editing (A)Cloning steps for the generation of the desired construct. (B) PLT2: YFP expression decreases over time after the estradiol induction (Image Courtesy: Wang et al., 2020)

Chapter 3: RESEARCH DESIGN AND METHODOLOGY

3.1 sgRNA and PAM sequence designing

To increase the binding specificity of sgRNAs, they should be compared with sequences of closely related ATG8 family of genes (*ATG8A*, *ATG8B*, *ATG8C*, *ATG8F*, *ATG8H*) and other non-redundant genes. The specificity of the Protospacer Adjacent Motif (PAM) sequence is of primary importance. The PAM sequence is typically a short, 2-6 nucleotide sequence that is located immediately adjacent to the target DNA sequence. The PAM sequence is essential for the Cas9 enzyme to recognize and bind to the target DNA. Cas9 cannot cleave DNA unless the target sequence is immediately followed by a PAM sequence. Different Cas9 enzymes have different PAM sequence requirements, which determines their target specificity. Hence, the relatedness of other closely related and non-redundant genes was compared with ATG8F using Clustal Omega and EMBOSS needle pairwise sequence alignment. EMBOSS needle was used to compare coding sequences (CDS) of two related genes and to find non-overlapping sites. Then, using Clustal Omega, multiple *ATG8F* gene was compared, and a specific region with the least similarity was selected for the PAM sequence. For the other isoforms of ATG8, see Supplementary table 6.1

ATG8F-TG1-gRT#+	**TGCTTACTTTTTCTTGTCGAGTTTTAGAGCTAGAAAT**
ATG8F-TG1-AtU3bT#-	**TCGACAAGAAAAAGTAAGCATGACCAATGTTGCTCC**
ATG8F-TG2-gRT#+	**TGCCCCACAGTCAGATCAGCGTTTTAGAGCTAGAAAT**
ATG8F-TG2-AtU3dT#-	**GCTGATCTGACTGTGGGGCATGACCAATGGTGCTTTG**
ATG8F-TG3-gRT#+	**TCCCGGCTGATCTGACTGTGGTTTTAGAGCTAGAAAT**
ATG8F-TG3-AtU6-1T#-	**CACAGTCAGATCAGCCGGGACAATCACTACTTCGTCT**
ATG8F-TG4-gRT#+	**GGAGAAAACACATTTGGATTGTTTTAGAGCTAGAAAT**
ATG8F-TG4-AtU6-29T#-	**AATCCAAATGTGTTTTCTCCCAATCTCTTAGTCGACT**

Table 3.1: Primers designed for the introduction of target sequences into sgRNA expression cassettes by overlapping PCR (Blue - Promoter/Scaffold; Red - Target)

The region next to it is assigned for the sgRNA sequence or target sequence generation of a sgRNA expression cassette containing a target sequence by overlapping PCR. Target sequences were selected using CRISPR-PLANT (https://www.genome.arizona.edu/crispr/) tool including PAM. These sequences were compared against the *Arabidopsis thaliana* genome sequence to confirm their targeting specificity in the genomes Secondary structure analysis of target-sgRNA sequences selected was carried out using RNA folding form (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3) program.

U-F	CTCCGTTTTACCTGTGGAATCG
gR-R	CGGAGGAAAATTCCATCCAC
Pps-GGL	TTCAGAggtctcTctcgACTAGTATGGAATCGGCAGCAAAGG
Pgs-GG2	AGCGTGggtctcGtcagggTCCATCCACTCCAAGCTC
Pps-GG2	TTCAGAggtctcT**c**tgacacTGGAATCGGCAGCAAAGG
Pgs-GG3	AGCGTGggtctcGtcttcacTCCATCCACTCCAAGCTC
Pps-GG3	TTCAGAggtctcTaagacttTGGAATCGGCAGCAAAGG
Pgs-GG4	AGCGTGggtctcGagtccttTCCATCCACTCCAAGCTC
Pps-GG4	TTCAGAggtctcTgactacaTGGAATCGGCAGCAAAGG
Pgs-GGR	AGCGTGggtctcGaccgACGCGTATCCATCCACTCCAAGCTC
PB-L	GCGCGCgGTctcGCTCGACTAGTATGG
PB-R	GCGCGCggtctcTACCGACGCGTATCC

Table 3.2: Primers designed for Golden Gate cloning of sgRNA expression cassettes

3.2 Generation of sgRNA expression cassette involves multiple PCR steps

We are generating four individual mutations hence, four different sgRNAs are required to target different regions of *ATG8F* and create multiple mutations. Unique Guide RNA promoters and their sgRNA scaffolds were amplified in two separate reaction conditions from their respective vector background- pYLsgRNA-AtU3b, pYLsgRNA-AtU3d, pYLsgRNA-AtU6-1, pYLsgRNA-AtU6-29. The promoter was amplified using U-F and AtU3/6#- (AtU3b, AtU3d, AtU6-1, AtU6-29) contains *ATG8F* short guide RNA target sequence flanked under the reaction condition 95°C pre-incubation for 1 minute followed by 95°C 10 seconds of denaturation 15 seconds for annealing primer in a gradient of 55 to 65 degree and 15 seconds for elongation at 68°C (Takara GXL Polymerase) for 35 cycles in an Eppendorff mastercycler. Scaffold was amplified using ATG8F-TG-gRT#+ (containing *ATG8F* short guide RNA Target sequence flanked) and gR-R under the same reaction condition under a gradient PCR. Four targets were amplified accordingly.

- **Vector backbones for IGE Cloning**

The vectors necessary for IGE cloning along with the *Addgene* catalogue numbers are as follows:

• *pYLsgRNA-AtU3b* (Plasmid #66198)

• *pYLsgRNA-AtU3d* (Plasmid #66200)

• *pYLsgRNA-AtU6-1* (Plasmid #66202)

• *pYLsgRNA-AtU6-29* (Plasmid #66203)

• *p2R3z-Bsa I-ccdB-Bsa I* (Plasmid #118389)

• *pFG7m34GW* (Plasmid #133747)

• *p221z-Cas9p-t35s* (Plasmid #118385)

• *p221z-Cas9p-tagRFP-t35s* (Plasmid #118386)

• *p221z-dCas9p-t35s* (Plasmid #118387)

3.3 Amplification of sgRNA promoter, scaffold, and Overlap PCR

Generation of IGE construct begins with amplifying individual sgRNA promoter and scaffold from their respective vector background (Target 1: *pYLsgRNA-AtU3b*, Target 2: *pYLsgRNA-AtU3d*, Target 3: *pYLsgRNA-AtU6-1*, Target 4: *pYLsgRNA-AtU6-29*) followed by overlapping the two individual fragments. Multiple gradient PCRs were performed to amplify all four targets and their corresponding DNA fragment length of promoter and scaffold was obtained and confirmed on Agarose (1% agarose; Sigma-Aldrich) gel electrophoresis. The individual promoter and scaffold sequences of four targets were then overlapped. After overlap PCR all fo3ur targets were confirmed using agarose gel electrophoresis.

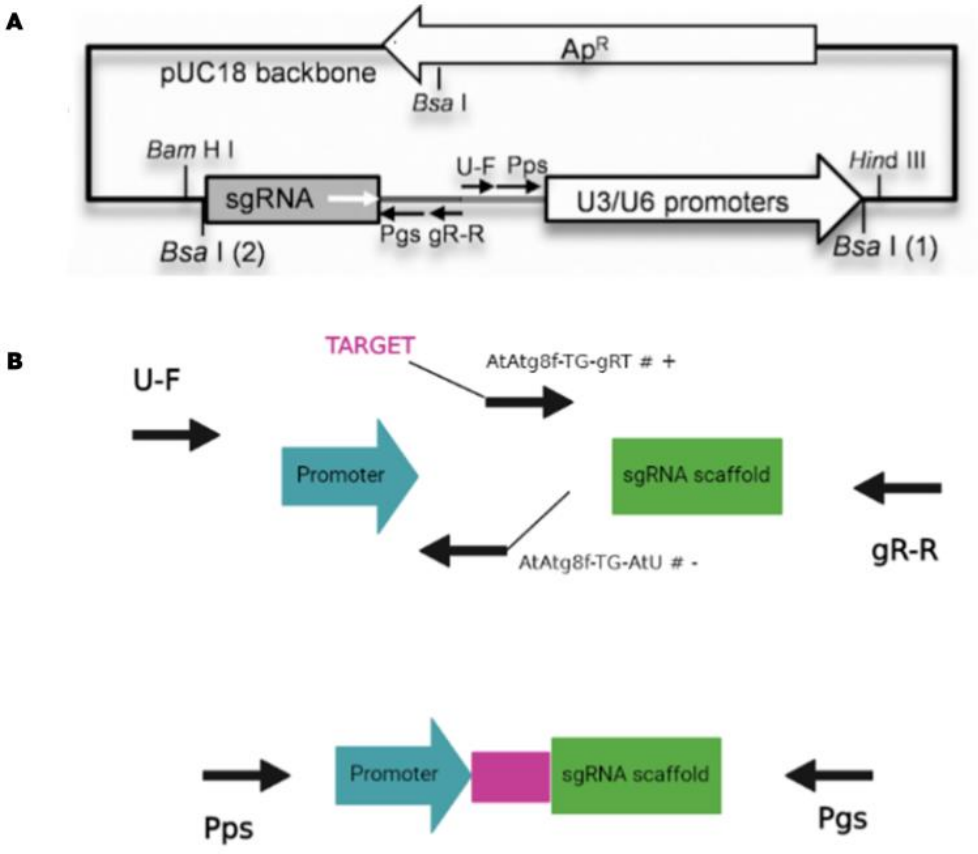


Fig. 3.1: (A) Vector representation of AtU3/6-gRNA (Image courtesy: Ma et al., 2015) (B) Graphical representation of individual amplification of promoter and scaffold with separate primers for each reaction (top). Overlap PCR of promoter and scaffold with chimeric primers (Bottom).

3.4 Golden gate assembly

Four sgRNA cassettes were cloned into the *p2R3z-Bsa I-ccdB-BsaI* entry vector by one-step Golden Gate cloning. The reaction was set up with 120 ng of *p2R3z-Bsa I-ccdB-BsaI,* 90 ng of purified PCR product of each sgRNA cassette, 2µl of T4 DNA Ligase buffer with 10Mm ATP, 1µl of BsaI-HF-V2 (20 U), 0.5µl of T4 DNA ligase (1000 U) and H2O to make up 20µl. The reaction mixture was incubated on a thermocycler under the following conditions: 37 °C for 5 min, 16 °C for 5 min for 30cycles, then 60 °C for 5 min and 80 °C for 5 min. The mixture was then used for *Escherichia coli* DH5α transformation.

3.5 Cloning of IGE constructs & Transformation of IGE constructs into *Arabidopsis thaliana*

In our case the construct (*pG1090:XVE::Cas9tagRFP:ATG8F* sgRNA cassette) that we have to generate has *1R4:pG1090I* as the estrogen inducible XVE based promoter in the first box (supplementary Fig. 6.). This promoter gets activated only upon induction with estradiol (a form of estrogen) in the growth medium. The second box consists of the Cas9 protein with RFP tag (supplementary Fig. 6.) (*p221z-CAS9p-TagRFP-t35s*, Addgene no. 118386) and the third box consists of the golden gate product i.e., *ATG8F* sgRNA cassette (supplementary Fig. 6.) (*p2R3z ATG8F* sgRNA cassette). To analyze whether the *ATG8F* gene knockout is purely due to Cas9 protein activity and to observe whether any other physiological changes are taking place upon the induction of estradiol in the growth medium, we also generated a control construct with dead Cas9 protein (*p221z-dCas9-t35s*; Addgene No. 118387) in the second box instead of the live Cas9 protein while keeping the first box and third box the same. These entry clones are recombined into a destination vector (*pFG7m34GW*; Addgene no.133747), which upon the successful LR recombination results in green fluorescence on the seed coat. The reaction mixture consisted of the three entry clones, the destination vector, and LR Clonase (Invitrogen); the setup was incubated at 25°C for 12 hours.

After the LR reaction, the construct was transformed into a DH5α strain of *E. coli* and incubated for twelve hours at 37°C (Bacterial resistance: Spectinomycin). The colonies obtained were screened for positives and the positive colonies were plasmid isolated (Macherey Nagel DNA purification kit). Then the obtained construct was electroporated into Agrobacterium-C58, which was plated and incubated at 29°C (bacterial resistance: rifampicin

and spectinomycin) for forty-eight hours. Single colonies were picked and cultured which were grown for 24 hours. The same process was done for the control construct (*pG1090:XVE::dCas9 t35s:ATG8F sgRNA* cassette) too.

The culture was used for *Agrobacterium*-mediated floral infiltration into *Arabidopsis thaliana*. We used *pATG8F::NG-gATG8F:3AT* in the wild-type background of *Arabidopsis thaliana* for the infiltration procedure. This background has been shown with ATG8F protein tagged with Neon Green reporter, it shows cyan fluorescence. Hence, transfecting the construct into this background will help us analyse the action of Cas9 protein upon induction with estradiol. Once the growth medium is induced the expression of ATG8F fades due to the activity of Cas9, this can be noted with the help of the tag associated with Cas9 (i.e., Neon Green fades due to knock out and RFP of the Cas9 intensifies due to induction). In the control experiment as the Cas9 is dead, the expression of ATG8F:NG will not fade even after estradiol induction.

Chapter 4: RESULTS AND DISCUSSION

The main objective of the project is to create a knockout of the gene *ATG8* and its isoforms using inducible CRISPR/Cas9 genome editing of *ATG8s* to modulate the expression of these genes during de novo organ regeneration in a tissue-specific and time-specific manner.

• Inducible Genome editing (IGE) construct cloning

• Agrobacterium-mediated Infiltration of the construct into *Arabidopsis thaliana* (Floral dip)

• Selection of seeds under UV (Construct Emit fluorescence-GFP)

• Analysis of the function of knockouts using best F1 lines.

4.1 Results

CRISPR/Cas9 uses a single or duplex of short RNA molecules which directs Cas9 protein to target the desired gene of interest Cas9 regulates DNA by sgRNA-DNA pairing between the 5' end of the leading sequence of the target DNA with guide RNA (gRNA) spacer (gRNA spacer is 20 base pair long). The four to six-base pair long protospacer adjacent motif (PAM) sequence helps the Cas9 recognize the target sequence region to nick by initiating nuclease activity. The nuclease activity of Cas9 is restricted to the regions identified by sgRNA, which has a PAM sequence next to the protospacer. The double-strand break (DSB) induced by nuclease activity are repaired by non-homologous end joining (NHEJ) or homologous recombination (HR) resulting in small or large chromosomal changes. These changes result in the loss of function mutations.

1. Designing of sgRNAs

sgRNAs and PAM sequences were chosen from the CRISPR PLANT tool. ATG8 belongs to a huge family of Autophagy genes consisting of *ATG1-ATG42*. Among these, *ATG8* is found to play a crucial role in regulating the autophagosome formation and transport. There are different isoforms of *ATG8; ATG8A, ATG8B, ATG8C, ATG8F, and ATG8H.* In this study, we are generating four knockouts within gene *ATG8F* and the development of knockouts for other isoforms are still in progress (Fig. 4.1).

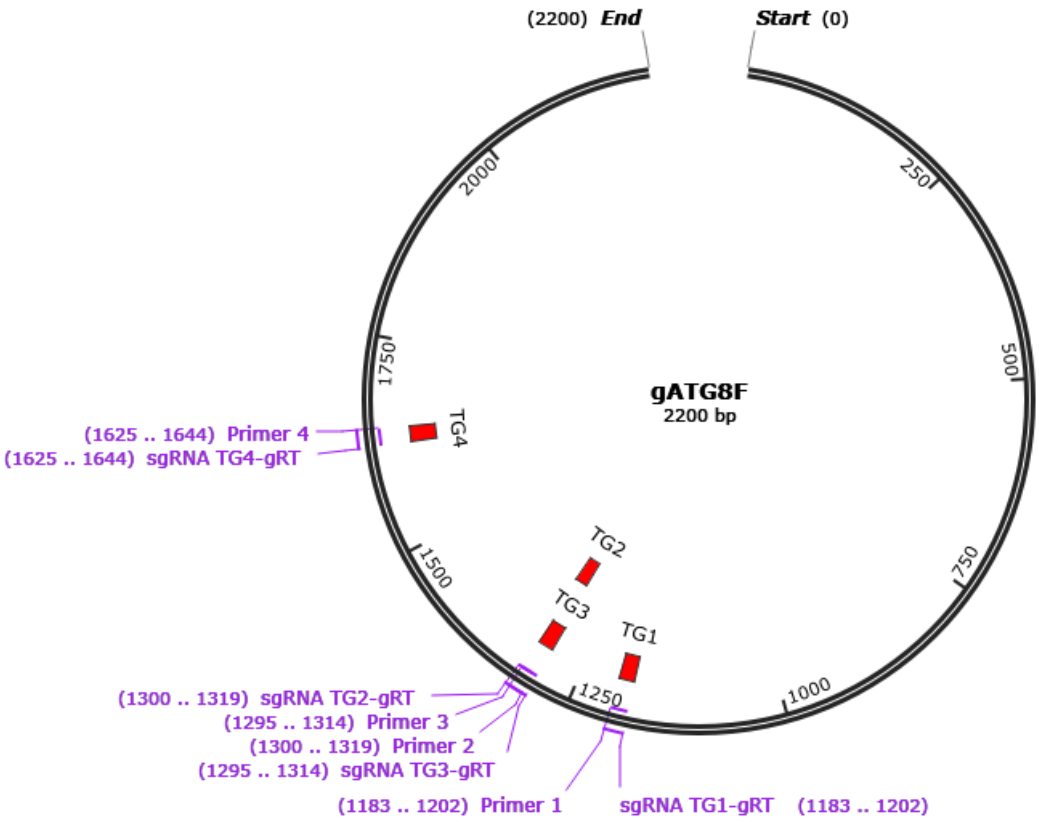


Fig. 4.1: sgRNA sequences and primers designed for genomic DNA of ATG8F

2. Amplification of sgRNA promoter, scaffold, and Overlap PCR

Generation of IGE construct begins with amplifying individual sgRNA promoter and scaffold from their respective vector background (*Target 1: pYLsgRNA-AtU3b, Target 2: pYLsgRNA-AtU3d, Target 3: pYLsgRNA-AtU6-1, Target 4: pYLsgRNA-AtU6-29*) followed by overlapping the two individual fragments. Multiple PCR was performed to amplify all four targets and their corresponding DNA fragment length of promoter and scaffold was obtained and confirmed on Agarose (1.8% agarose; Sigma-Aldrich) gel electrophoresis. The individual promoter and scaffold sequences of four targets were then overlapped. After overlap PCR all four targets were confirmed using agarose gel electrophoresis.

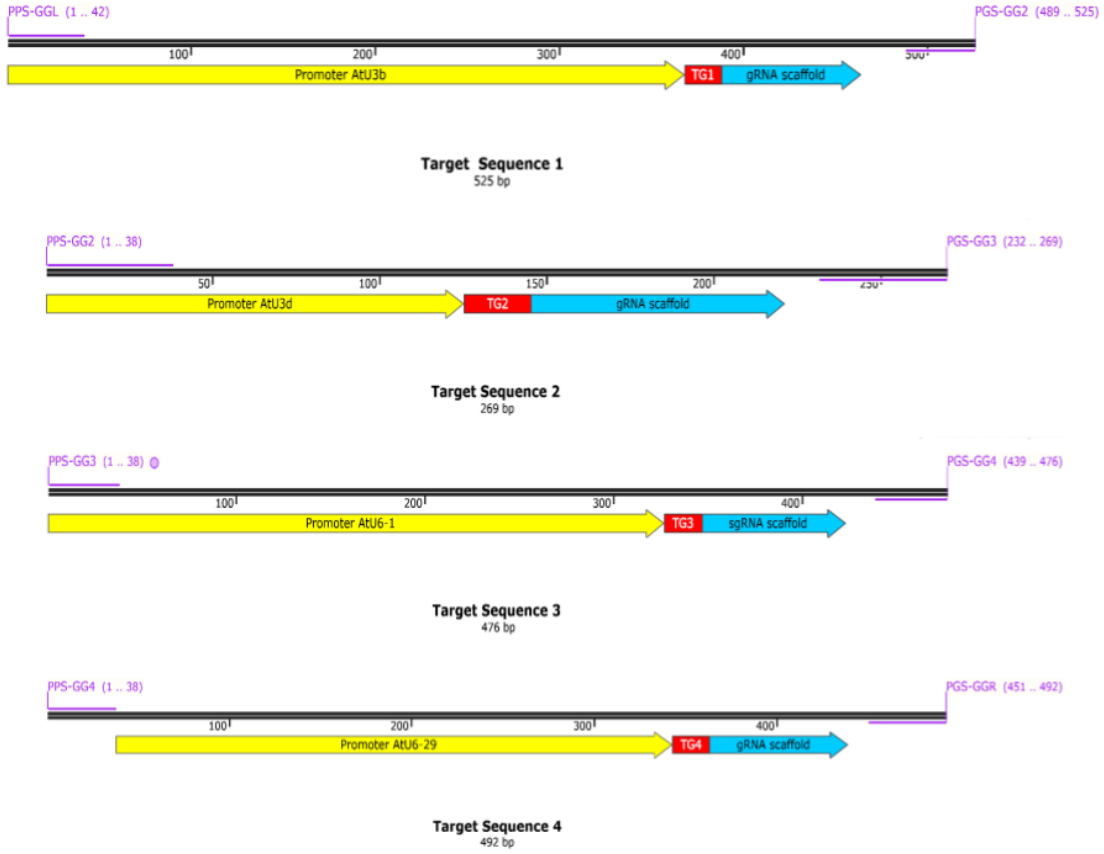
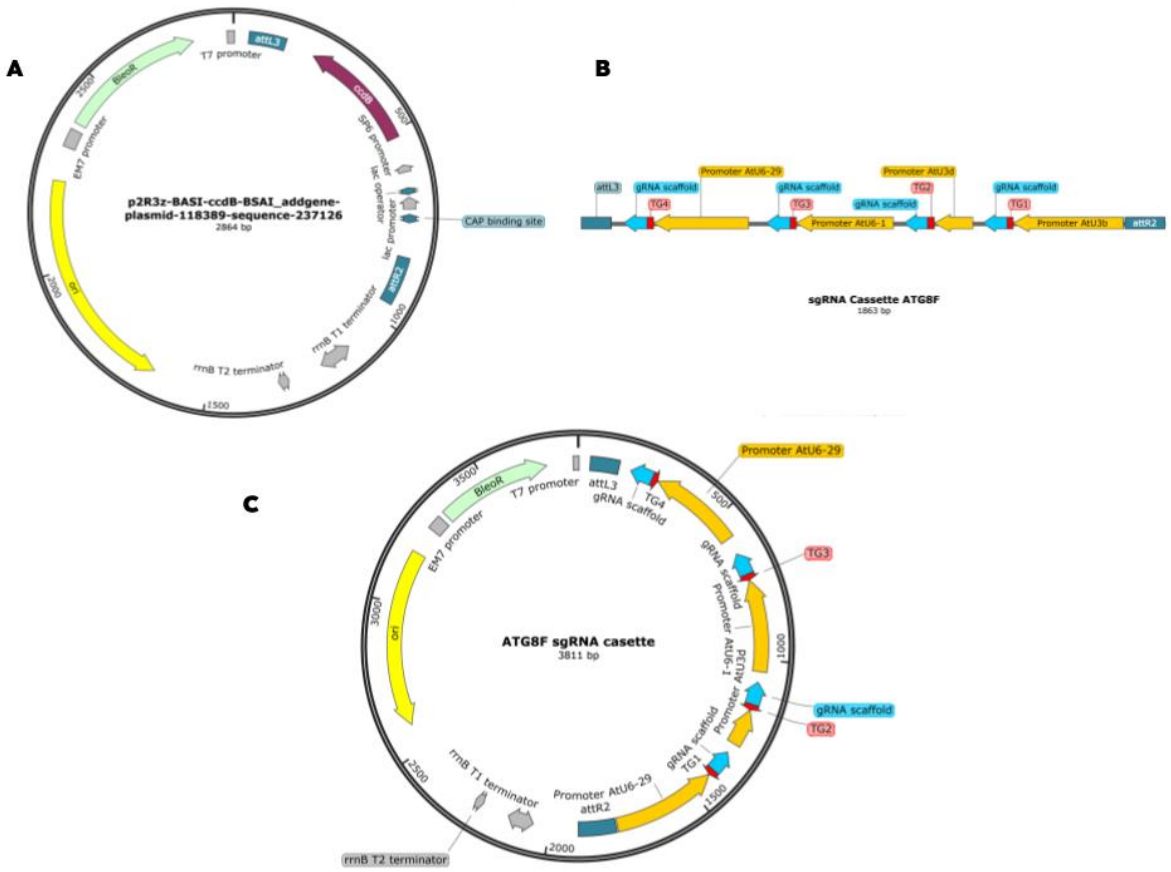


Fig. 4.2: Target sequences after Bsa1 Flank PCRs (Simulated via Snapgene)

3. Golden gate assembly

Golden gate cloning or golden gate assembly allows the simultaneous integration of multiple DNA fragments into a single vector using type II restriction enzyme and T4 DNA ligase. This single reaction involves both digestion and ligation simultaneously. Golden gate assembly exploits the ability of type IIS restriction endonucleases to cleave DNA outside of the recognition sequence. The inserts and cloning vectors are designed in such a way that the type IIS enzymes recognition site is distally located from the cleavage site, such that enzymes can remove their sequence of recognition from the assembly to make way for the ligation. Removing their own cut sites from the DNA fragment prevents the further activity of the enzyme. This property also prevents the self-ligation of the vector and helps in achieving complementarity. Ligation happens in an orderly manner because, after digestion with the restriction enzyme, the remaining sequence in the vector backbone will have a complementary DNA fragment hence both will ligate together. This DNA fragment will also be complementary to the next DNA fragment and so on the assembly will ligate.

In our case, all the overlap PCR products (four fragments) and the vector backbone (*p2R3z-Bsa I-ccdB-BsaI*) to which these fragments are to be ligated are added to a reaction mixture together (Fig. 4.3). The overlapped fragments need to be assembled into a single vector. Golden gate assembly helps to assemble multiple fragments with the help of a type IIs restriction enzyme. We used the BSAI-HF-V2 enzyme for the digestion and T4 DNA ligase for ligation. The reaction product was transformed into DH5α and the positives were confirmed with the help of restriction digestion using ApaLI enzyme and agarose gel electrophoresis (Fig. 4.3 (E)). Further, to double-check whether the construct is inserted successfully or not it was then amplified using PB-L and PB-R as primers (binds exactly on the cassette region) to test the nonspecific amplification. Taq polymerase (Takara GXL) along with Taq buffer was used to amplify the product under the conditions of 95°C ten seconds denaturation followed by 62°C annealing and elongation for one minute and 55 seconds as the total size of the product is 1863bp. Amplicon was confirmed with no other band could be sent for sequencing in the future to confirm if the desired product is obtained or not.



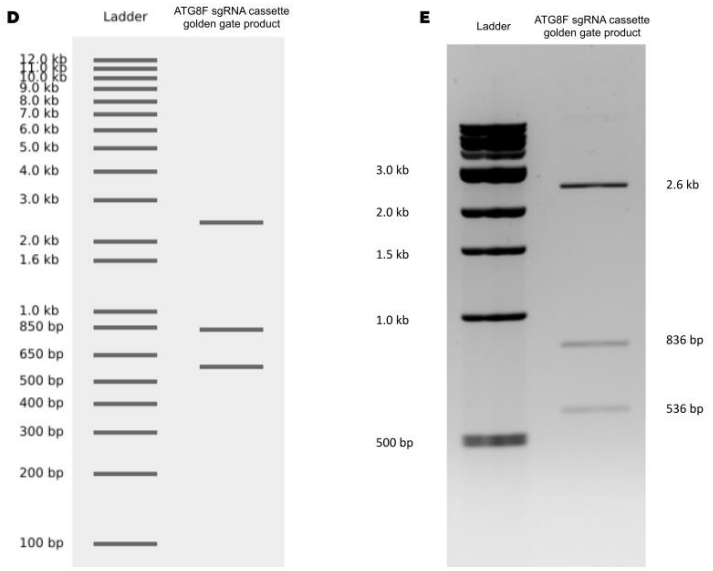


Fig. 4.3: Golden Gate Cloning. (A) Plasmid map for the empty vector p2R3z-Bsa I-ccdB-Bsa (B) sgRNA Cassette for golden gate cloning (C) ATG8F sgRNA cassette insert after the golden gate. (D) Virtual digest for the final product using ApaI1 enzyme. (E) Agarose gel image for the restriction digestion

5. Multisite Gateway cloning and inducible genome editing

To generate an inducible Cas9 construct we need to use the Multisite Gateway inducible cloning system for *Arabidopsis thaliana*. Multisite gateway cloning is a recombination technology developed inspired by the bacteriophage that integrates its genomic DNA into its host bacterial genome. It allows the integration of multiple DNA fragments into one construct in a single cloning step. This system combines the inducible promoter (first box), the gene of interest (second box), and terminator/reporter lines (third box) as gateway entry clones with binary destination vectors in a multisite LR Clonase reaction. The benefit of using an inducible promoter is that we can express the action of the construct when desired. The cell type-specific nature of this system also benefits, as deleterious effects of ectopic gene expression can be avoided. An estrogen-inducible XVE-based promoter is used here. This inducible system has very low levels of constitutive and baseline activation also, Estrogen inducible system causes very minimal physiological or developmental anomalies.

After successful cloning of the golden gate product i.e., *ATG8F* sgRNA cassette, Multisite gateway cloning was utilized to clone the inducible promoter (*1R4 pG1090I:XVE*; first box)

with Cas9 protein (*p221z-CAS9p-TagRFP-t35s*; second box) and the golden gate assembly (*p2R3z-ATG8F* sgRNA Cassette; third box) using LR Clonase enzyme. The recombined product (*pG1090:XVE::Cas9tagRFP:ATG8F*-sgRNA cassette) (Fig. 4.4) was transformed into DH5α later positives were screened using restriction digestion with ApaLI enzyme and screening via agarose gel electrophoresis. The control experiment with dead Cas9 protein (*p221z-dCAS9p-t35s*; second box) was also done in the same manner. After the Multisite gateway reaction, the control construct (*pG1090:XVE::dCas9:t35s: ATG8F* sgRNA cassette) was also transformed into DH5α and screened for positive colonies using ApaLI as a restriction enzyme.

The successfully cloned constructs (*pG1090:XVE::Cas9tagRFP: ATG8F* sgRNA cassette, *pG1090:XVE::dCas9p:t35s: ATG8F* sgRNA cassette) with Cas9 and dCas9 were then electroporated into C58 strain of *Agrobacterium tumefaciens* which was then cultured and used for infiltration into wildtype *pATG8F::ATG8F- NG* background *Arabidopsis thaliana*. After screening for positives for GFP, these seeds will be germinated on Kanamycin plates to check for the actual positives.

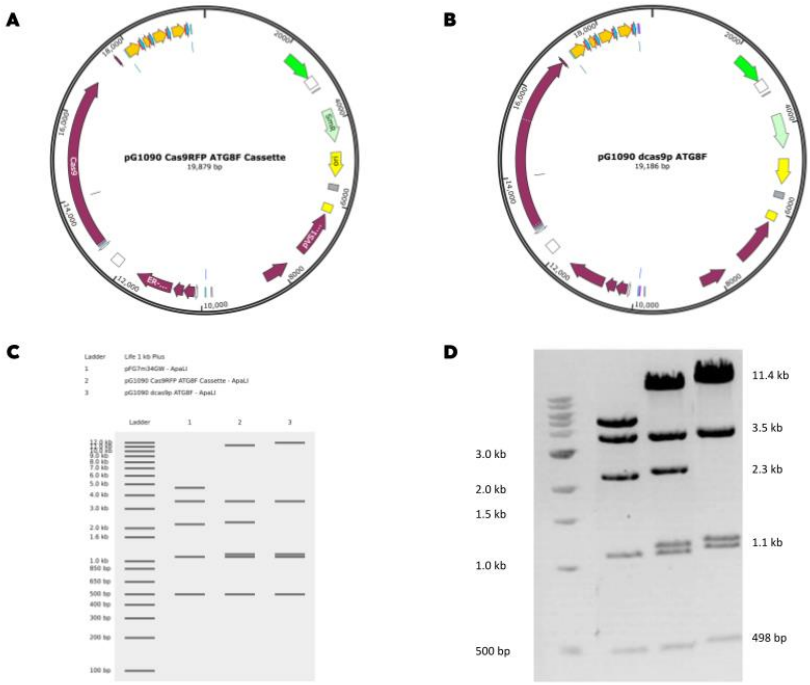


Fig. 4.4: Multisite Gateway Cloning. (A) pG1090:XVE::Cas9tagRFP: ATG8F sgRNA cassette (B) pG1090:XVE::dCas9p:t35s: ATG8F sgRNA cassette (C) Virtual digest of the plasmids along with empty vector with ApaI1 enzyme simulated using Benchling (D) Agarose gel image of the digestion with ApaI1 enzyme

4.2 Conclusion

Recent studies from the lab have shown that *de novo* root regeneration from the leaf petiole in *Arabidopsis* is affected by disturbing the function of autophagy genes through pharmacological, genetic, and molecular perturbations (Akansha Ganguly, *unpublished*). This shows the possibility of the existence of pathways for wound-induced regeneration for which autophagy is very essential.

Out of hundreds of proteins that are involved in the regulation of autophagy, ATG8 is essential throughout the process, while other proteins are transiently expressed. Therefore, developing a knockout for *ATG8F*, an isoform of *ATG8* protein, would help us to understand the role of autophagy in *de novo* organogenesis. The experiment would also throw light on the pathways that could potentially involve the interplay of the PLETHORA (PLT) transcription factors with ATGs. *De novo* root formation is expected to either reduce drastically or not show up at all in these mutants.

My major thesis revolved around generating *ATG8F* knockout. As we needed to analyze how autophagy affects *de novo* root regeneration at a particular time point after cutting the leaf and placing it on a medium. Normal CRISPR/Cas9 system was not feasible as the system knocks out the gene throughout the developmental phase. We needed an inducible CRISPR Cas9 system, which can activate Cas9 activity upon induction. An inducible promoter (pG1090I) was used for inducing the activity of Cas9 upon induction in the estradiol medium. Primarily we had to design and construct multiple (four) sgRNA cassettes necessary for guiding Cas9 toward the nick site by using different PCR methods (Overlap PCR, Flank PCR, and Golden gate assembly). After successful generation of the *p2R3z ATG8F* cassette multi-site gateway reaction was used to clone the three entry clones: inducible promoter (*1R4 pG1090I:XVE*, first box), Cas9 protein (*p221z Cas9tagRFP t35s*, second box) and sgRNA cassette (*p2R3z ATG8F* cassette, third box) using LR Clonase into the destination vector (pFG7m34GW). Then the construct was transformed into DH5α and later into *Arabidopsis thaliana* via *Agrobacterium tumefaciens* (C58) mediated infiltration-control construct with dead Cas9 (*p221z dCas9 t35s*) was also transformed the same way to verify that *ATG8F* gene knockout is exclusively due to the activity of Cas9. The transformation was done onto a *col ATG8F: NG* background to visualize the action of Cas9 upon induction (NG decreases as the

ATG8F gets degraded due to Cas9 activity and this activity can be visualized as the Cas9 protein is tagged with RFP). The plants are kept in the incubator for drying.

Further on dried seeds were collected and visualized under a fluorescent microscope for positive selection as the destination vector shows green fluorescence on the seed coat. The positive seeds will be run on regeneration assay during which the plants will be exposed to estradiol for a brief period of time to initiate the activity of Cas9. This will allow us to elucidate the role of ATG8F in *de novo* root regeneration and in turn help us contemplate how autophagy could affect *de novo* root regeneration.

4.3 Discussion

Recent studies have shown the robust nature of the CRISPR/Cas9 system for targeting multiple genes. Binary vectors have been shown to initiate gene knockout in a tissue-specific fashion only at desired time when the Cas9p gene is regulated by inducible promoters and combined with various plant-selectable marker genes and specific sgRNAs (Ma *et al.*, 2015; Wang *et al.*, 2020). The current cloning techniques serve to enhance mutation rates and minimise off-target effects by efficiently producing numerous sgRNA expression cassettes. Several studies have shown that higher GC content results in increased efficiency in mutation with biallelic mutations being the strongest followed by homozygous and heterozygous mutations.

Mutations which are homology-directed in question could have come from a mutation that was initially created by the Double Strand Break with Non-Homologous End Joining pathway in the specified location of a chromosome. Then, the Cas9/sgRNA complex cleaves the complete allelic target site of a different chromosome and repairs it using the first mutant allele as the template, resulting in a homozygous mutation in the locus. Cas9-mediated gene editing has less off-target effects than other commonly used methods. However, one inaccuracy of the system is that gRNA-Cas9 complex's imprecise recognition of the PAM site's compatibility with the gRNA spacer. By matching the PAM sequence as uniquely as possible, this could be minimised.

De novo root formation from leaves of different species has been in use for the propagation of those species for a very long time. However, not much is known about the cellular, subcellular,

molecular, and genetic factors that influence and modulate the root formation. The most intriguing questions include: How is the cell integrity maintained after such intensive wounding? How does the mechanical stimulation induce the dedifferentiation of a specific cell type which then forms a root primordium? Is there a specific time for which the mechanical stimulation should be provided post-injury? If so, is this time window conserved in other species? The root could be part of an emergent property shown by the plant. But most of the younger leaves were observed to get decolorized and degraded after 15 days. Then why are the cellular energetics directed towards making a root, but not a shoot? The leaf cells near the petiole are seen to have expanded very much after placing the detached leaf on the medium. Could this be due to endoreduplication? Endoreduplication has been shown to have cell wall loosening effects. Could this affect the development of the root primordia through a mechanical conflict between neighbouring cells? These are all questions that I am really interested in and would like to explore more. I hope the knockout line for *ATG8F* would help in finding answers for at least some of the questions listed above.

EXPLORING THE VARIATIONS IN THE *DE NOVO* ORGAN REGENERATION POTENTIAL ACROSS THE PLANT SPECIES

The first land vascular plant discovered on earth dates back to 425 million years ago (Harrison and Morris, 2018), and it is no wonder that over time they have evolved high plasticity during development. One of the important factors that make plants different from other species on earth is the indefinite growth they possess. The meristems on the root and shoot apex and on lateral sides as well makes this possible. Plants also show high regenerative capacity when compared to higher-order animals for which the regenerative ability is restricted to a certain lineage of cells (Birnbaum and Sánchez Alvarado, 2008). Plants could easily build the entire body plan from a small part of the tissue when provided with necessary cues. The method of propagation of plants via tissue culture exploits the same principle for the generation of an entire plant from a small explant using the exogenous application of phytohormones. A huge fraction of molecular and genetic works in plants is centred around *Arabidopsis thaliana*, which is considered the perfect model organism considering its short life span, high number of seeds, ease of growth, etc. Regeneration studies in *A. thaliana* have uncovered many mechanisms and pathways in which different proteins and phytohormones act to regulate organogenesis from an explant (Benková *et al.*, 2003; Kakani *et al.*, 2009; Tsugeki *et al.*, 2009). Many assays for these studies have been discovered and standardized in the past years (Mathew and Prasad, 2021).

The touch-dependent *de novo* root regeneration is a nuance observation and needs to be studied in detail to explore the mechanisms involved in the process. To check the universality of the observation, the same experiment was carried out on more than a dozen species from the families like *Asteraceae, Malvaceae, Solanaceae, Fabaceae,* etc. Among these, a plant called *Sonchus oleraceus* from the family *Asteraceae* showed some interesting features regarding the root formation from the petiole. The variations from the expected result were the key motivation for looking at the plant in detail.

The leaves of *Sonchus oleraceus* aka common sow thistle were collected from the greenhouse surroundings. They were placed in both vertical and horizontal positions, keeping the cut end of the petiole in touch with the soil, mixed with full-strength MS medium. Formation of the root was observed in most of the samples (some got decayed probably because of fungal infections) by the 7th day of planting. The leaves were taken out to document and placed back inside the soil for another 8 days. On the 15th day of observation, multiple shoots (8 to 10)

were coming out of the base of the petiole that was placed vertically. Similar observations were not found in the ones that were placed horizontally. We designed further experiments to check if there is an effect of gravity or if is it enough to have continuous mechanical stress near the petiole to form multiple shoots as well as roots, because when vertically placed, the weight of the leaf blade is exerting a force on itself at the cut end of the petiole, and the intuition was that this could be the driving force for the generation of multiple shoots in addition to normal root growth. So, to introduce continuous mechanical stress, we inserted a pin near the cut end of the petiole and placed the leaves horizontally. After 15 days, it was observed that multiple shoots were forming from both sides of the site where the pin got inserted.

Fig. 5.1: Formation of multiple roots and shoots from Sonchus leaf petiole. (A) The primary root emerges after 7dpi. (B) The complete root system along with new shoots at 15 dpi. (C) The leaf placed vertically forms multiple shoots after 15 dpi. (D) Zoomed-in image of (C). (E) A leaf placed horizontally with an additional mechanical injury forms multiple shoots on either side of the injury after 15 dpi.

To explore the basis for such aberrant responses to touch-dependent DNRR, we decided to look for the conserved genetic and molecular pathways that might have a role in the regulation of regeneration. Using the algorithms provided by NCBI BLAST, CLUSTAL OMEGA, and EMBOSS NEEDLE, we analysed the conservation of specific factors required for regeneration both at the level of transcription and translation. Degenerate primers were designed for *plt* and *wox* genes which play an important role during the regeneration and root meristem formation.

These were further used to quantify the expression levels of the mRNAs, to check if they are being down or upregulated during the process of DNRR in *S. oleraceus*.

Degenerate primer designing

PLT and *WOX* proteins are essential and found to be upregulated in the case of *de novo* root regeneration. To evaluate whether this is the same for other plants during touch-dependent DNRR, we decided to go for a qRTPCR analysis of Differentially Expressed genes that are known to play a role in DNRR. Therefore, we designed degenerate primers considering the conservation of sequences of *plt* and *wox* genes across *Arabidopsis* and the species of family *Asteraceae* for which the genomic sequence is available and accessible through NCBI.
The details of the primers that were designed and ordered are listed below in Table 5.1.

Common_plt1	GATATGAGGCTCATCTATGGG	GC = 48%, T_m = 53°C, 21 bp	GCTTTATCTTCTTTGTCATATCC	GC = 35%, T_m = 52°C, 23 bp
Asteraceae_plt2	CATAGATGGACAGGAAGATAT	GC = 38%, T_m = 50°C, 21 bp	GTGCAGCCATATCATAAG	GC = 44%, T_m = 50°C, 18 bp
Asteraceae_plt4	GGACAAAGAACTTCTATTTACC	GC = 36%, T_m = 51°C, 22 bp	CTTCTTTATCATAACCTCCC	GC = 40%, T_m = 50°C, 20 bp
Common_plt7	GGACAAAGAACTTCAATTTATCG	GC = 35%, T_m = 53°C, 23 bp	AAGTACACTTGACGTCCTTTTC	GC = 41%, T_m = 55°C, 22 bp
	AGGCATCATCAACAAGG	GC = 47%, T_m = 52°C, 17 bp	GCCTCTGCTGCTTCC	GC = 67%, T_m = 54°C, 15 bp
Common_wox5	GATGGAATCCAACGGTGG	GC = 56%, T_m = 54°C, 18 bp	CCAATAGAAAACATTCTTGCTCT	GC = 35%, T_m = 53°C, 23 bp
Asteraceae_wox5	GTGTGGTCGGTGGAA	GC = 60%, T_m = 52°C, 15 bp	CATTCTTGCTTTCGATCTT	GC = 37%, T_m = 50°C, 19 bp
Asteraceae_wox11	GAACCAGTTAGGTCAAGAT	GC = 42%, T_m = 52°C, 19 bp	CCGAATTTCTCAAGCAG	GC = 47%, T_m = 50°C, 17 bp

Table 5.1: Degenerate primers designed using BLAST and CLUSTAL OMEGA

To check the fidelity of the primers that were designed and ordered, first, we needed good quality RNA from the plant sample that could be used to create cDNA which could further be processed for downstream analysis. All the primers were designed in such a way that the region of amplification has a length of around 150 bp. For efficient cDNA synthesis, the PrimeScript™ RT reagent Kit (Perfect Real Time) of catalogue #RR037A from TAKARA was used. The components of the kit are listed below.

5X PrimeScript Buffer (for Real Time)	400 µl
PrimeScript RT Enzyme Mix	100 µl
Oligo dT Primer	100 µl
Random6mers	400 µl
RNase Free dH2O	1 ml
EASY Dilution (for Real Time PCR)	1 ml

The protocol for the cat. #RR037A was followed and the PCR conditions were set according to the primer length and polymerase conditions. Amplicons were compared using a 100-base pair ladder. The primers were analysed for specificity using 3-step PCR amplifications and the respective amplicons for all 3 primers of plt7, wox5, and tubulin respectively are as shown below.

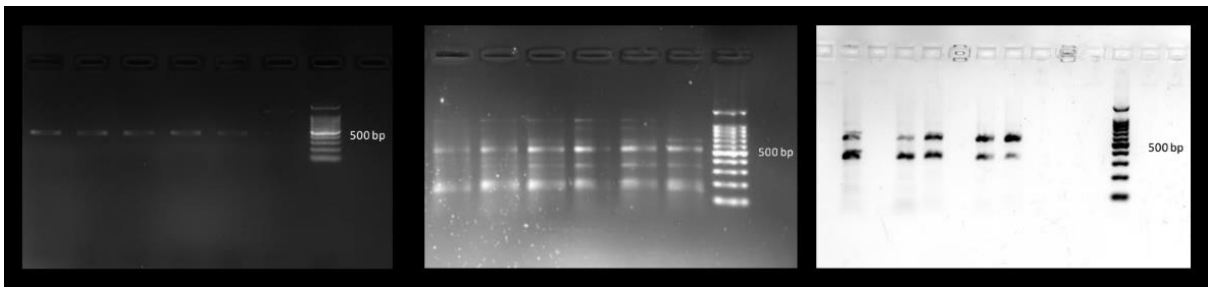


Fig. 5.2: PCR Amplification of PLT7, WOX5, and Tubulin in Sonchus

Standardization of tissue culture and agrobacterium-mediated plant transformation

Auxin is an important phytohormone that plays a crucial role in plant growth and development. Therefore, it becomes important to know the localization of auxin to study the dynamics of the events that happen during DNRR (Xu, 2018). But the cellular and fluorescent protein-tagged markers are available for the model organism extensively, whereas, for any other system to work on, this has to be developed manually. So, our next step was to create a transgenic that could show the levels of auxin that could be visualized and quantified via fluorescence. The DR5-Venus system is an efficient reporter for studying auxin dynamics. Hence, we tried to insert the plasmid with DR5-Venus via Agrobacterium-mediated transformation, into the plant system.

The transformation protocols required co-culturing with the callus because direct application of Agrobacterium on floral meristem as done in *Arabidopsis* (Floral dip method, supplementary material) has very less probability to work in *Sonchus*. Hence, the transformation using co-culture with callus was implemented which didn't come out as expected. The survival of the plant parts/callus on the medium after the infection was challenging. Washing with the antibiotic cefotaxime to kill off excess *Agrobacterium* didn't make much difference. More nuanced techniques like microinjection and protoplast co-culture should also be implemented to get efficient transformation and the production of the first-ever transgenic *Sonchus oleraceus*. The composition of media and amount of phytohormones used is as listed below. Callusing takes almost 18 days, which is longer, compared to *Arabidopsis*.2,4-D at higher concentrations, and Benzyl aminopurine (BAP) was found to be an effective and optimum growth condition for callus. Callusing was observed to continue for more than 60 days once it was formed from explants.

Trial No.	Salt Used	Total Volume	Hormones and concentration	Observation
1	MS	250 ml	2,4-D (1mg/ml) - 125 ul Kinetin (1 mg/ml) - 12.5 ul	No proliferation from petiole, root and leaf parts
2	MS	250 ml	IBA (1 mg/ml) - 125 ul	1) Lateral roots formed from root sections 2) Callus formed after 3 weeks of incubation
3	MS	125 ml	IBA (1mg/ml) - 125 ul BAP (0.5 mg/ml) - 12.5 ul	Lateral roots formed for the ones transferred from IBA only media, kept in light
4	B-5	250 ml	2,4-D (1mg/ml) - 500 ul BAP (0.5 mg/ml) - 25 ul	Callus formation

Table 5.2: Tissue culture standardization with different media and phytohormones

Results and Discussion

The plant *S. oleraceus* is an amazing model system to study wound-induced regenerative responses. The novelty of the system is that it shows deviations from normal wound healing and *de novo* root regeneration when the system is subjected to multiple injuries. Unlike Arabidopsis, the leaf is able to form multiple shoots upon injury-mediated touch dependent regeneration. Apart from the fact that the plant is an allotetraploid(Hsieh *et al.*, 1972), the lack of any genetic information about the system is a major hindrance to understanding its varied regenerative behaviours. But to study the system in a much more sophisticated manner, one has to begin from the scratch and explore the molecular and genetic aspects of the system that could be the key to uncovering novel pathways and nuanced insights into cellular regeneration.

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SUPPLEMENTARY DATA

ATG8B-TG1-gRT#+	CAGAGGATTAGAAAGCTTGAGTTTTAGAGCTAGAAAT
ATG8B-TG1-AtU3bT#-	TCAAGCTTTCTAATCCTCTGTGACCAATGTTGCTCC
ATG8B-TG2-gRT#+	TAAAATCCTTTACCAGTTGGGTTTTAGAGCTAGAAAT
ATG8B-TG2-AtU3dT#-	CCAACTGGTAAAGGATTTTATGACCAATGGTGCTTTG
ATG8B-TG3-gRT#+	TTCAAGCTTTCTAATCCTCTGTTTTAGAGCTAGAAAT
ATG8B-TG3-AtU6-1T#-	AGAGGATTAGAAAGCTTGAACAATCACTACTTCGTCT
ATG8B-TG4-gRT#+	AAGAACACAAAGACGAAGACGTTTTAGAGCTAGAAAT
ATG8B-TG4-AtU6-29T#-	GTCTTCGTCTTTGTGTTCTTCAATCTCTTAGTCGACT
ATG8C-TG1-gRT#+	GGGTTTTGGTAATGGCAGAGGTTTTAGAGCTAGAAAT
ATG8C-TG1-AtU3bT#-	CTCTGCCATTACCAAAACCCTGACCAATGTTGCTCC
ATG8C-TG2-gRT#+	GAAGTTGTGTGTTTACCAGTGTTTTAGAGCTAGAAAT
ATG8C-TG2-AtU3dT#-	ACTGGTAAACACACAACTTCTGACCAATGGTGCTTTG
ATG8C-TG3-gRT#+	AATTGAATCTTCTCGCATCAGTTTTAGAGCTAGAAAT
ATG8C-TG3-AtU6-1T#-	TGATGCGAGAAGATTCAATTCAATCACTACTTCGTCT
ATG8C-TG4-gRT#+	TCACTTCTTCTTGTCGATATGTTTTAGAGCTAGAAAT
ATG8C-TG4-AtU6-29T#-	ATATCGACAAGAAGAAGTGACAATCTCTTAGTCGACT
ATG8H-TG1-gRT#+	ACAGTTAGACAGGTTACACTGTTTTAGAGCTAGAAAT
ATG8H-TG1-AtU3bT#-	AGTGTAACCTGTCTAACTGTTGACCAATGTTGCTCC
ATG8H-TG2-gRT#+	TTGTTAGCCGAAAGTTTTCTGTTTTAGAGCTAGAAAT
ATG8H-TG2-AtU3dT#-	AGAAAACTTTCGGCTAACAATGACCAATGGTGCTTTG
ATG8H-TG3-gRT#+	GGATATAGATGAGAGATTGAGTTTTAGAGCTAGAAAT
ATG8H-TG3-AtU6-1T#-	TCAATCTCTCATCTATATCCCAATCACTACTTCGTCT
ATG8H-TG4-gRT#+	ATTTGTTTATTGTAGATACTGTTTTAGAGCTAGAAAT
ATG8H-TG4-AtU6-29T#-	AGTATCTACAATAAACAAATCAATCTCTTAGTCGACT

Table 6.1: Primers designed for the introduction of target sequences into sgRNA expression cassettes by overlapping PCR (Blue - Promoter/Scaffold; Red - Target

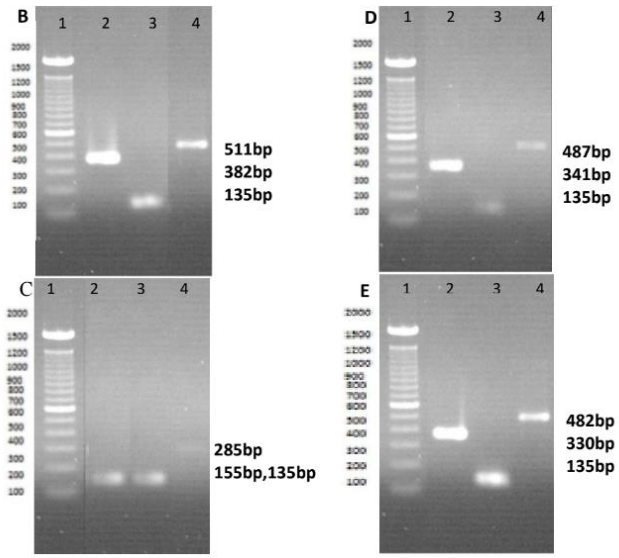
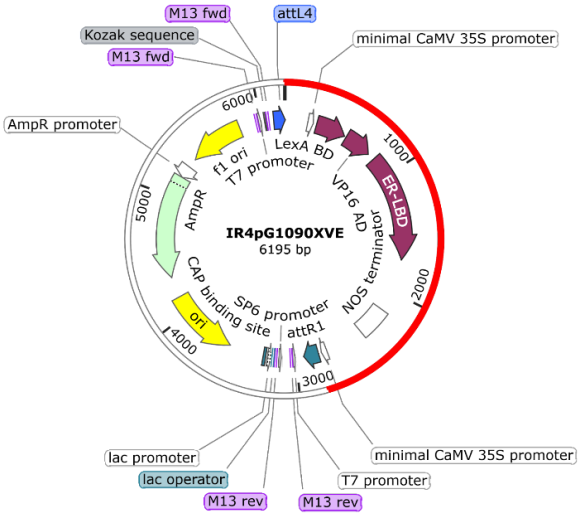


Fig. 6.2: Amplicons for Overlap and Flank PCRs

Multisite Gateway Cloning: Pg1090:XVE::Cas9 tag RFP: ATG8F



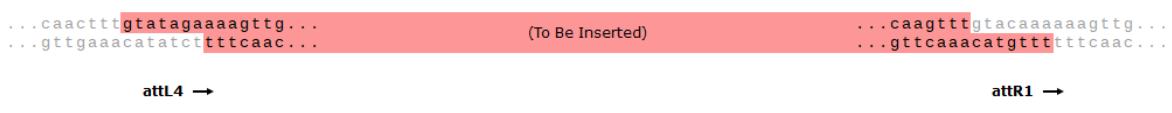


Fig. 6.3:First Box

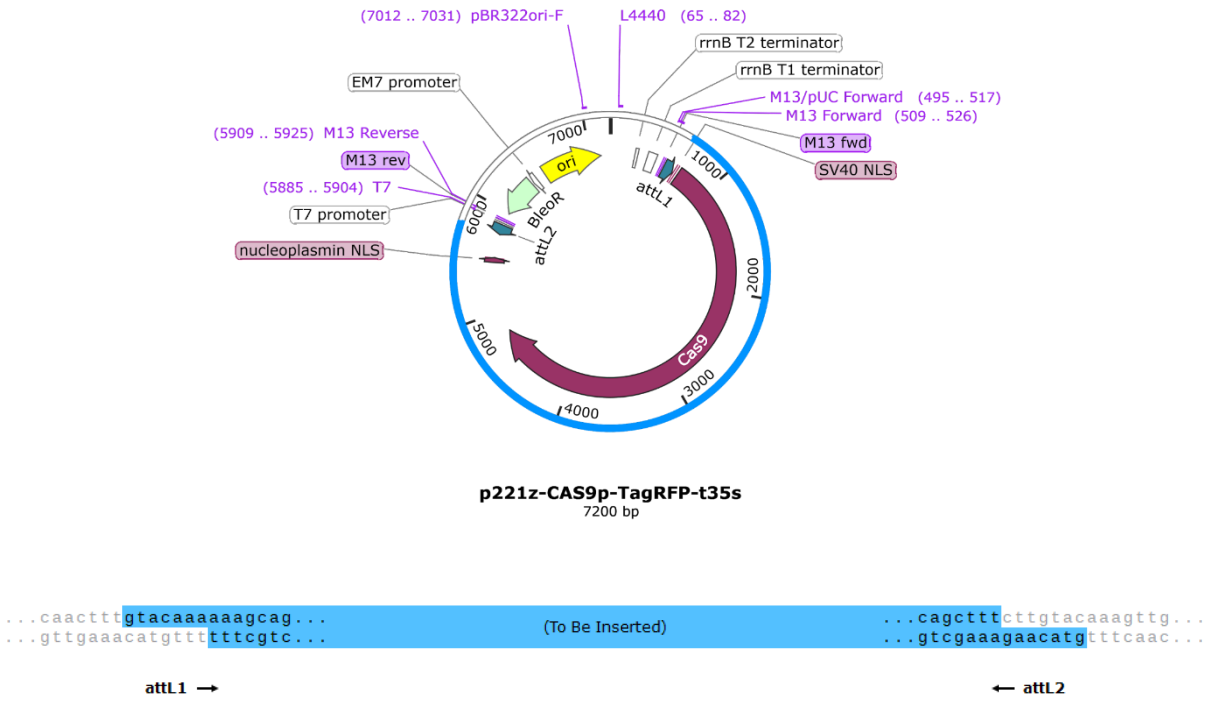


Fig. 6.4: Second Box

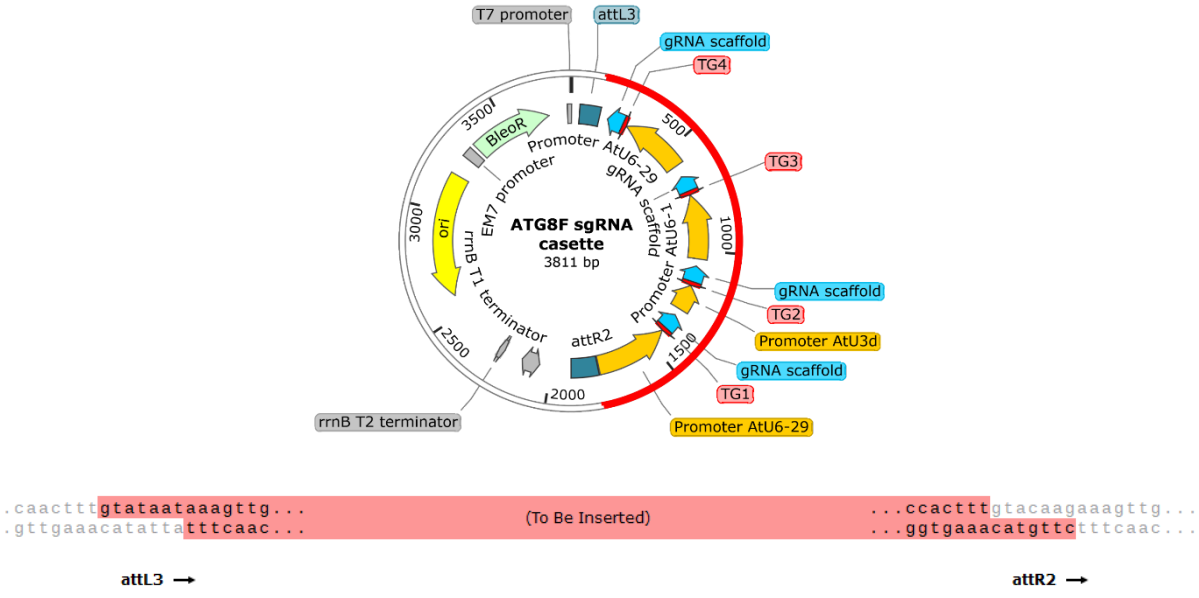


Fig. 6.5: Third Box

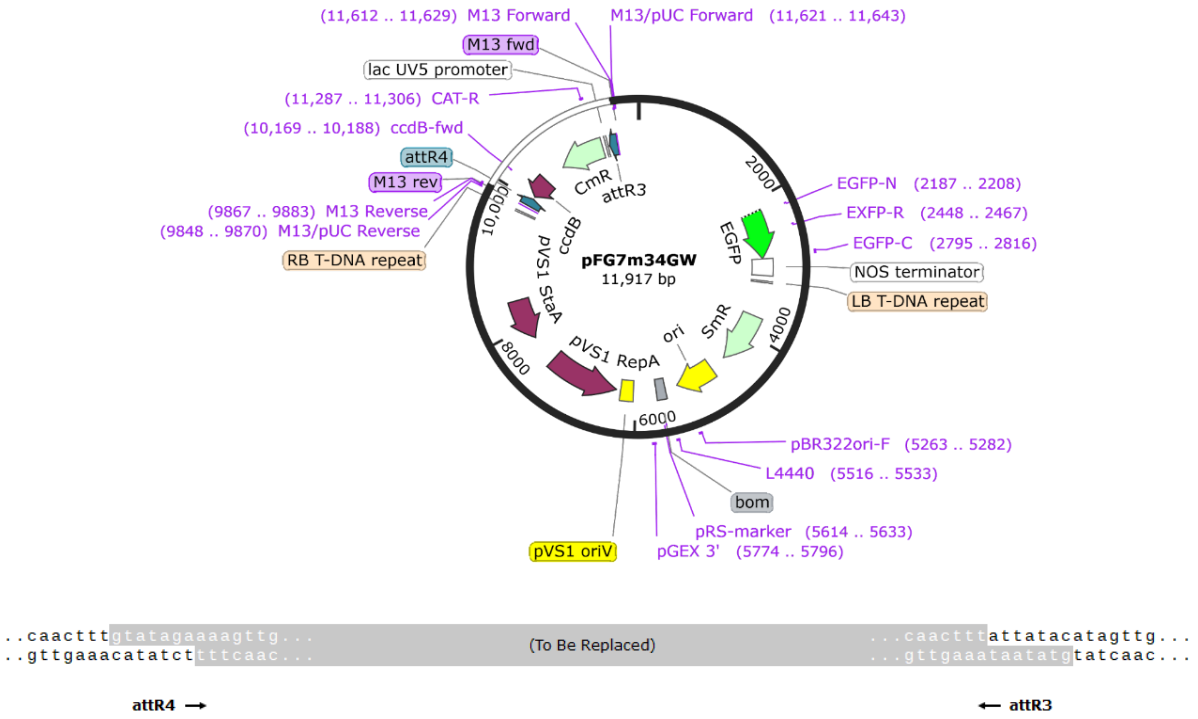
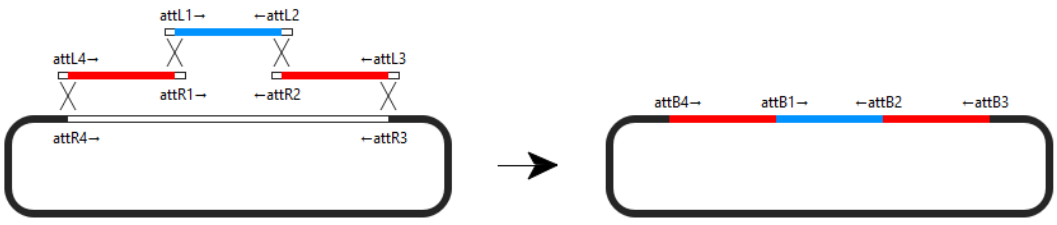


Fig. 6.6: Destination Vector

Multisite Gateway Cloning: Mechanism



Final Product for Multisite Gateway Cloning : pG1090:XVE::Cas9tagRFP:ATG8F

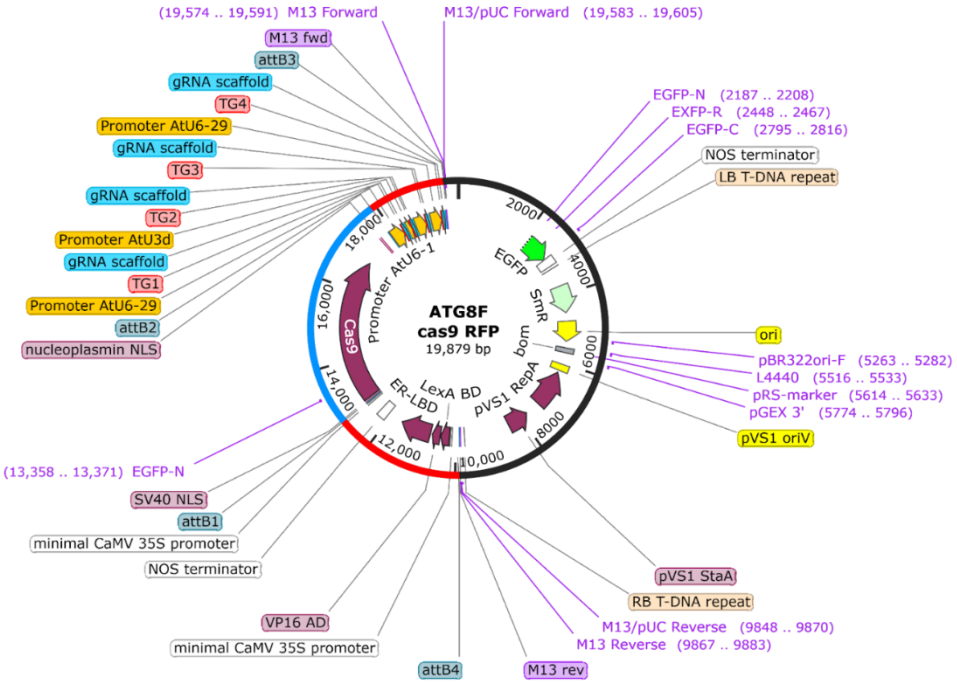


Fig. 6.7: pG1090i::Cas9tagRFP: ATG8F sgRNA cassette

2. Multisite Gateway Cloning for pG1090:XVE::dCas9p: ATG8F

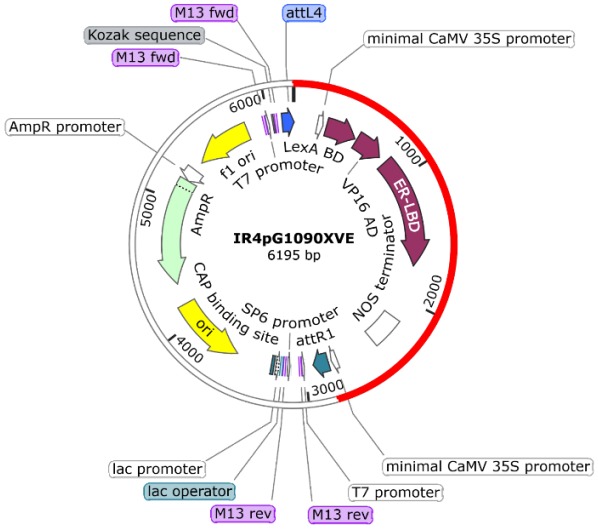


Fig. 6.8: First Box

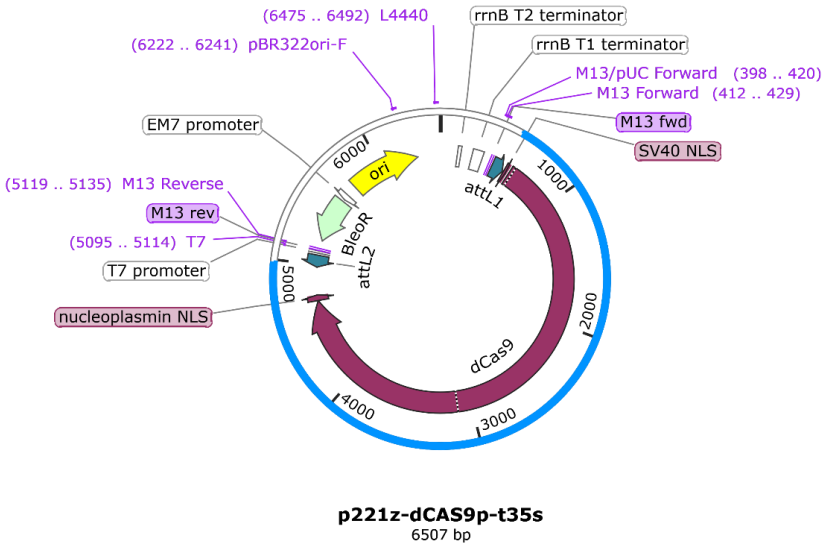


Fig. 6.9: Second Box

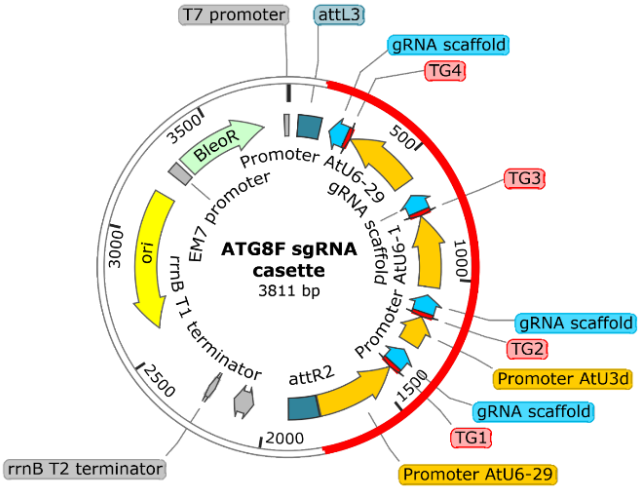


Fig. 6.10: Third Box

Final product for Multisite Gateway Cloning for pG1090:XVE::dCas9p: ATG8F

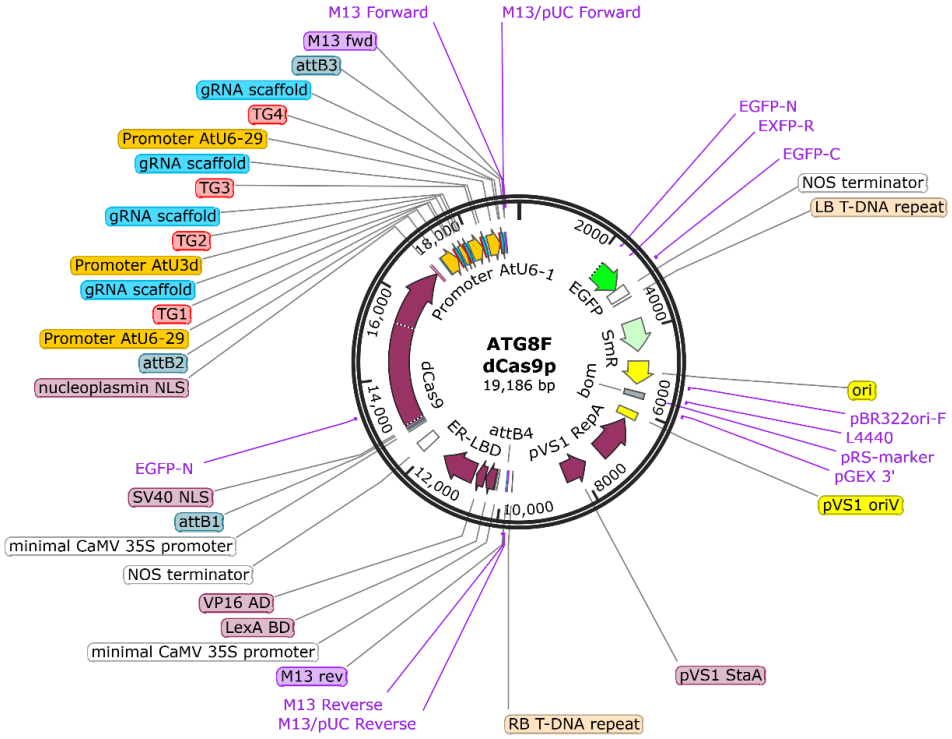


Fig. 6.11:pG1090i::dCas9p:t35s: ATG8F sgRNA cassette