MOLECULAR CLONING OF A CONSTRUCT FOR TARGETED *Rec*A GENE DISRUPTION VIA HOMOLOGOUS RECOMBINATION IN *Agrobacterium tumefaciens*

By

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ABSTRACT

MOLECULAR CLONING OF A CONSTRUCT FOR TARGETED *Rec*A GENE DISRUPTION VIA HOMOLOGOUS RECOMBINATION IN *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens is a cornerstone of plant genetic engineering and serves as the primary vector for delivering transgenes into a wide variety of plant species. However, the efficiency of this process is compromised by the bacterium's native homologous recombination system, which is mediated by the RecA protein. Functional RecA promotes unintended rearrangements and deletions of the transferred DNA (T-DNA) within the bacterial cell, leading to integrated transgenes that are fragmented, rearranged, or incomplete. This reduces the precision and effectiveness of *Agrobacterium*-mediated transformation (AMT) and necessitates screening of excessive numbers of transformants to recover a few with the correct construct, making the process inefficient and costly. This study aimed to address this limitation by constructing a targeted gene disruption vector to create a stable *recA*-deficient strain of *A. tumefaciens*. The strategy involved engineering a plasmid that upon introduction into *Agrobacterium* would insertionally disrupt the function chromosomal *recA* gene with a (*cat*)-*rep*A cassette via a double-crossover homologous recombination event. To achieve this, the N-terminal and C-terminal regions of *rec*A were amplified by polymerase chain reaction (PCR)

using *A. tumefaciens* genomic DNA as a template. These fragments were then sequentially cloned into the pASK-KO suicide vector flanking the chloramphenicol resistance (*cat*)-*repA* cassette. The resulting construct, pASK-NrecA-cat-repA-CrecA, was assembled and propagated in *Escherichia coli* TOP10 cells. Putative recombinant clones were selected on kanamycin-containing media and validated using a combination of colony PCR, restriction enzyme digestion, and Sanger sequencing, which confirmed 100% identity with the expected plasmid sequence. The successful construction of this vector is a critical first step. Its subsequent introduction into *A. tumefaciens* is expected to generate a mutant strain with a disrupted *recA* locus. This engineered strain should exhibit significantly enhanced T-DNA plasmid stability, thereby minimizing rearrangements and ultimately increasing AMT fidelity and efficiency. The development of such specialized strains has significant potential to streamline plant biotechnology workflows, reducing the time and resources required to generate transgenic plants with intact functional genes.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotation and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman (UTAR) or other institutions.

Student's signature

APPROVAL SHEET

This final year project report entitled "**MOLECULAR CLONING OF A CONSTRUCT FOR TARGETED RECA GENE DISRUPTION VIA HOMOLOGOUS RECOMBINATION IN *Agrobacterium tumefaciens***" was prepared by VICTORIA LAW and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Honours) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by: **Date:**

02.09.2025

Prof. Wong Hann Ling

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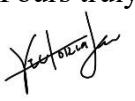
Final Year Project Thesis Dissemination Consent Form

I hereby agree that dissemination or disclosure, in any forms or means, in whole or in part of my FYP thesis MOLECULAR CLONING OF A CONSTRUCT FOR TARGETED RECA GENE DISRUPTION VIA HOMOLOGOUS RECOMBINATION IN *Agrobacterium tumefaciens* shall be made only with consent of the project supervisor (Prof. Wong Hann Ling) and co-supervisor (name).

Yours truly,



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LIST OF ABBREVIATIONS

µL	Microliter
2X YT	2X Yeast-Tryptone
AMT	*Agrobacterium*-mediated transformation
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
bp	Base pair
$CaCl_2$	Calcium chloride
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
g	Relative centrifugal force
GFP	Green fluorescent protein
kb	Kilobase
$MgCl_2$	Magnesium chloride
mL	Milliliter
MMS	Methyl methanesulfonate
PCR	Polymerase chain reaction
ssDNA	single-stranded DNA
T-DNA	Transferred DNA

Ti plasmids	Tumor-inducing plasmid
A. tumefaciens	*Agrobacterium tumefaciens*
Vir	Virulence
YFP	Yellow Fluorescent Protein

CHAPTER 1

INTRODUCTION

1.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a Gram-negative, rod-shaped, soil-borne bacterium known to cause crown gall disease in plants (Sunday et al., 2024). However, during the last century, significant discoveries have revealed the unique properties of *Agrobacterium*, which has changed its perception from being merely a plant pathogen to a powerful tool in genetic engineering. In the 1940s, scientist Armin Braun demonstrated that once a plant was infected with *Agrobacterium*, tumors continued to grow even when the bacterium was no longer present (White and Braun, 1941). In addition, he recognized that the resulting plant tumor cells could grow in a medium lacking plant hormones. This indicates that *Agrobacterium* altered the properties of plant cells, and Braun termed this factor the 'tumor-inducing principle' (TIP) (Braun, 1947; Braun and Mandle, 1948). After 30 years of research, scientists confirmed that TIP is a piece of DNA from a large plasmid in *Agrobacterium,* known as a tumor-inducing Ti plasmid. In addition, the Ti plasmid contains a section of DNA called T-DNA, which is excised and delivered into plant cells. In addition, the transfer of T-DNA is mediated by virulence genes in the Ti plasmid. As *Agrobacterium* senses signalling molecules at the wound site on plant cells, vir genes are switched on. This allows single-stranded T-DNA to enter plant cells via a type IV secretion system (Nester, 2015).

Once T-DNA is integrated into plant chromosomes, it causes the production of auxin and cytokinin, which leads to uncontrolled cell division. Opine synthesis is also expressed to provide a nutrient source for the bacterium. The ability of *Agrobacterium* to transfer any DNA inserted between the T-DNA borders of the Ti plasmid has made it a cornerstone of plant genetic engineering (Nester, 2015).

1.2 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation (AMT) has been widely used in plant genetic engineering, owing to its efficiency, simplicity, and relatively low cost. Traditional transformation methods, such as protoplasts, take a long time to regenerate plants and have low efficiency. In addition, the microprojectile bombardment method used to introduce foreign genes into plants often results in multiple DNA copy insertions, DNA rearrangements, and genetic disruption. Owing to its advantageous properties, AMT is widely applied in the production of transgenic plants and genetically modified crops.

AMT can be classified into two types: stable and transient. During stable transformation, T-DNA is permanently integrated into the host genome. This expression was inherited in subsequent generations. For instance, root explant transformation and floral dip method are often used in *Arabidopsis thaliana*. Stable transformation is high-throughput and eliminates the need for time-consuming tissue cultures. On the other hand, transient transformation allows short-term gene expression without genome integration. Moreover, it is useful

in rapid functional studies to assess promoter behavior, protein function, and gene silencing (Hwang, Yu and Lai, 2017).

Furthermore, the development of binary vectors further increases the versatility of AMT for different purposes. Additional features, such as selectable markers and reporter genes, allow for quantification of the transformed cells. In addition, AMT has been shown to successfully transform non-plant organisms including sea urchins, algae, and human cells. This demonstrates the broad potential of AMT as a gene delivery tool (Hwang, Yu and Lai, 2017).

1.3 *rec*A gene, and its role in *Agrobacterium*

RecA plays multiple roles in bacteria. These include: (i) initiating the SOS response to DNA damage, (ii) enabling mutagenesis during stress, and (iii) carrying out homologous recombination to repair DNA lesions. When DNA damage occurs, RecA binds to single-stranded DNA, forming a filament. The filament then triggers the cleavage of the LexA repressor. Subsequently, the SOS response was activated for DNA repair. In addition, RepA is also required for SOS mutagenesis. In SOS mutagenesis, RepA activates DNA polymerase V to bypass the DNA lesions that block replication. Most importantly, the RepA protein plays a role in DNA repair through homologous recombination, which uses energy from ATP hydrolysis (Cox, 2007).

In molecular cloning, the stability of the plasmid DNA within a host cell is crucial. Since *rec*A encodes RecA recombinase, it can catalyze homologous recombination that promotes the formation of plasmid multimers, deletions, or unwanted rearrangement between repeated sequences. These recombination events can lead to plasmid loss, especially when using plasmids with high copy numbers. In addition, a functional RecA recombinase can mediate undesirable intra- or intermolecular homologous recombination within or between a binary vector, Ti plasmid, and chromosomal DNA. These events may cause plasmid or chromosomal rearrangements and deletions, thereby reducing transformation efficiency or leading to the transfer of rearranged transgenes into the plant host cell. Therefore, *rec*A-deficient strains are preferred over *rec*A^+ strains to minimize unwanted recombination and to ensure the maintenance of cloned DNA in the host cell (Yang et al., 2022).

1.4 Targeted gene disruption via homologous recombination.

Homologous recombination was chosen as the method for constructing the *rec*A knockout strain because of its precise, scar-free replacement of *rec*A compared to random mutagenesis or transposon insertions (Aliu et al., 2024). Moreover, a more efficient recombineering system using shorter flanking homology arms was recently developed, confirming that homologous recombination is a reliable and reproducible method for genome engineering (Bian et al., 2022). Despite the popularity of CRISPR/Cas-based methods, their application in AMT is

limited owing to off-target risks and host-specific challenges (Rodrigues et al., 2020).

1.5 Significance of study

This project demonstrated its significance by establishing a methodology to disrupt the *rec*A gene in *A. tumefaciens* C58C1 via homologous recombination. RecA knockout enhanced plasmid stability and improved AMT efficiency in *A. tumefaciens*. In addition, it will allow future studies regarding the role of *rec*A in DNA repair pathways, plasmid stability, and T-DNA integration. This project can serve as a foundation for the future development of a more efficient vector by combining the ΔrecA mutant with other genetic modifications to build next-generation *Agrobacterium* strains tailored for biotechnological and agricultural applications.

1.6 Objectives

The main objective of this study was to generate a gene construct for disrupting *rec*A in the *A. tumefaciens* C58C1 strain via homologous recombination.

The specific objectives of this project are as follows:

(i) To clone N terminus fragment of *rec*A into pASK-KO plasmid

(ii) To clone C terminus fragment of *rec*A into the sequencing verified recombinant clone pASK-NrecA-repA

(iii) To verify the final recombinant construct pASK-NrecA-repA-CrecA for successful cloning of N and C-*rec*A via DNA sequencing

CHAPTER 2

LITERATURE REVIEW

2.1 Role of *rec*A gene in DNA repair

The *rec*A gene is important for bacterial growth. It encodes a recombinase that participates in DNA lesion repair, including collapsed replication forks and double-strand breaks. The repair mechanism employs homology-directed repair (HDR), which involves searching for homologous double-stranded DNA (dsDNA) to be used as a template to repair the break. RecA recombinase binds to single-stranded DNA (ssDNA) at the break, forming a helical filament (Figure 2.1). RecA uses adenosine triphosphate (ATP) to search for homologous DNA sequences. Once a homologous sequence is found, ssDNA is exchanged with intact DNA, producing a heteroduplex (Figure 2.2). Subsequently, the DNA repair machinery takes over to fill in the missing gap by DNA polymerase (Del Val et al., 2019).

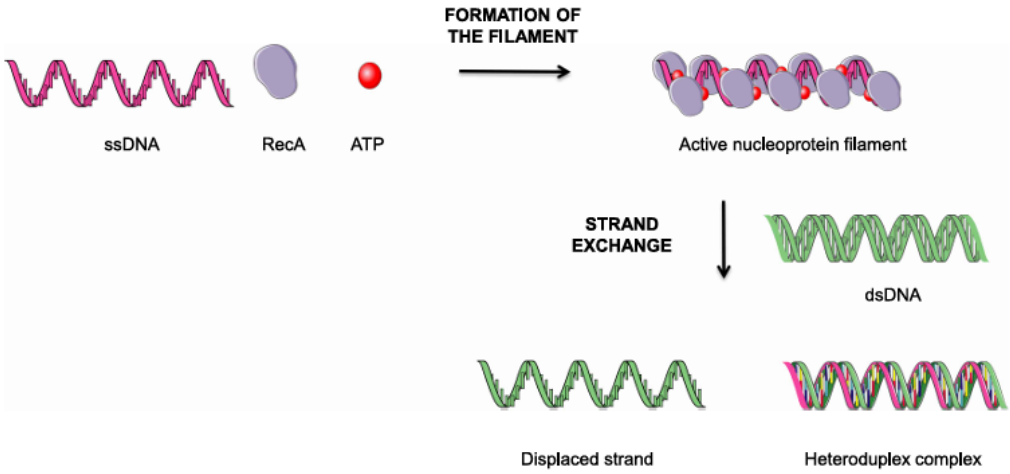


Figure 2.1 RecA activity in homologous recombination. RecA protein binds to ssDNA to form helical nucleoprotein. This filament further aids to search for homologous dsDNA (shown in green) (Del Val et al., 2019)

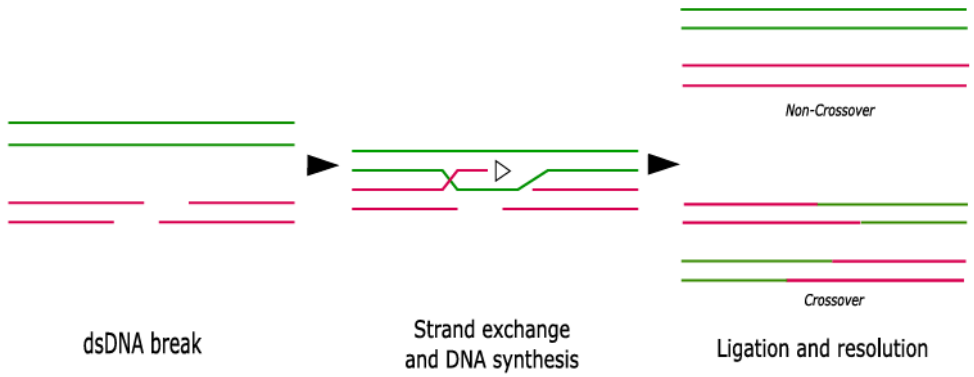


Figure 2.2 Process of homologous recombination. The RecA protein look for a homologous dsDNA (shown in green) as a template for synthesizing DNA. A ssDNA-RecA filament is formed, and the cross over between ssDNA (in red) and homologous dsDNA occur to exchange DNA strand. The resulting molecule creates two possible molecules: non-crossover (upper diagram) or crossover DNA product (lower diagram) (Del Val et al., 2019).

2.2 Seminal research of *rec*A in *Agrobacterium tumefaciens*

In a study by Farrand, O'Morchoe and McCutchan (1989), the *rec*A gene in *Agrobacterium tumefaciens* C58 was identified through functional complementation in *rec*A-deficient *E. coli*. A cosmid library was constructed using *A. tumefaciens* NT-1 genomic DNA and cloned into a wide-host-range cosmid vector, which was then introduced into the *E. coli* HB101 strain with the *rec*A mutation. The results showed that a gene from a cosmid library complements the function of *rec*A in *E. coli* with *rec*A deficiency. Complementary DNA confers resistance to the DNA-damaging agent methyl methanesulfonate (MMS) and UV radiation. This suggests that the complementary DNA carried a functional *recA homolog in A. tumefaciens*. The location of *rec*A was further detected by subcloning the fragment of DNA into a smaller plasmid and Tn3HoHo1 lacZ transposon mutagenesis was used to map the fragment that contained *rec*A. Finally, it was discovered that the *rec*A is approximately 1.3 kb within the fragment and the direction of transcription was established. Furthermore, the study tested the *rec*A function in the native host *Agrobacterium* by inserting an erythromycin resistance cassette in the coding region to replace the wild-type allele via marker-exchange mutagenesis. This produces a *rec*A knockout strain, UIA143, which has disrupted *recA* and is sensitive to UV radiation and MMS, as well as with the loss of ability to carry out homologous recombination. However, the *recA-deficient* UIA143 strain retained its capability for conjugation, transformation, and tumorigenicity. This further demonstrates that *rec*A recombination and other *rec*A associated functions are not essential for T-DNA transfer and integration (Farrand, O'Morchoe & McCutchan, 1989). Moreover, this study also justifies the use of

A. tumefaciens C58-derived strains over other strains. First, the C58 lineage is transformable and many auxotrophic mutants have been isolated. Additionally, a chromosomal map of C58 has been constructed to ease the gene manipulation process (Wood, 2001). In contrast, strain Ach5 had a low transformation rate, few useful mutants, and no chromosomal map.

2.3 Application of the *rep*A gene

Binary Ti plasmids are systems of two plasmids used in *Agrobacterium*-mediated plant transformation to transfer specific DNA segments and T-DNA into plant cells. In the binary vector system, one of the plasmids has T-DNA that carries the gene of interest and selectable markers, while the other plasmid, called helper plasmid, carries virulence (*vir*) genes that facilitate the transfer of T-DNA. Among other binary vectors, the pGreen series binary vector has been widely used because of its compact design, high cloning efficiency, and flexibility in molecular cloning (Hellens et al., 2000; Pratt, Knoblauch & Kunz, 2020; Chang et al., 2025). Before the pGreen series plasmids, cloning efficiency was lower for the larger plasmids. Additionally, different transformation protocols require different selection markers or reporter genes. The pGreen series binary vectors have several advantages over other binary vectors. Besides being smaller in size and high copy number, it also consists of multiple cloning sites for convenient cloning. Furthermore, selectable markers and reporter genes can be included (Hellens et al., 2000). Despite pGreen vectors have smaller plasmid size upon removal of replication initiation gene (repA) and mobilization gene (Mob), pGreen vectors lost replication function in *A. tumefaciens* (Hellens

et al., 2000; Alok et al., 2021). Therefore, pGreen vectors require another helper plasmid pSOUP to provide pSa-RepA gene for replication support in *A. tumefaciens* (Hellens et al., 2000; Yap et al., 2025). In other words, pGreen vectors have to co-resident with pSOUP in the host. Hence, this increases the complexity of the strain construct as well as plasmid stability (Hellens et al., 2000; Fabio Pasin et al., 2017). In this experiment, the *rep*A cassette was used to replace *rec*A in *A. tumefaciens*. Ultimately, pSOUP would no longer be required to co-maintain with pGreen vectors upon the successful integration of *rep*A gene into chromosomal DNA of *A. tumefaciens* because the host strain can express the replication function *in trans*. The integration of *rep*A cassette into *rec*A locus play a role in disruption of *rec*A gene in *Agrobacterium* and reduce reliance on pSOUP to be used with pGreen vector.

2.4 Phenotypic test on *rec*A-deficient strain

In the study by Renzette and Sandler (2008), UV irradiation assay was used to test the UV sensitivity of *rec*A knockout strains. The cultures were exposed to UV at a dose of 10 J m^{-2} for 20 min. The result of UV survival test showed that *rec*A mutants exhibited low survival rate of less than 0.01 %. In contrast, the *rec*A+ strain has a survival rate of 76%. This result indicates that the lack of functional RecA protein makes the cells highly sensitive to UV damage. Furthermore, SOS induction was quantified using GFP fluorescent driven by sulAp::gfp reporter construct. The *rec*A-deficient also showed reduced SOS response compared to the wild type. Finally, the recombination ability was also

evaluated, in which the conjugational recombination frequency 3.8×10^{-5} were significantly lower than 8.3% in recA deletion mutant. In short, this study demonstrated UV irradiation as a functional test for *rec*A.

2.5 Incorporation of negative selection marker to prevent overgrowth in *Agrobacterium*-mediated plant transformation

Overgrowth of *Agrobacterium* in *Agrobacterium*-mediated plant transformation has been a major factor that led to reduced transformation rates in plants (Ramadhan et al., 2022). The common practices involve using antibiotics such as cefotaxime and carbenicillin on *Agrobacterium*-mediated transformed plants. However, this method is not ideal as it decrease plant transformation efficiency. One of the strategies to overcome this overgrowth issue is by manipulating the gene construct of *Agrobacterium* plasmid for higher plant transformation rate. In 2016, Liu et al. has developed a *rec*A mutant in disarmed *A. tumefaciens* strain GV2260 using allelic-exchange approach. A suicide vector and *sac*B gene was used for counterselection. The suicide vector was built with upstream and downstream flanking sequences of *rec*A, including a *sac*B-*sac*R cassette that functions to disrupt *rec*A gene and allow sucrose counterselection. The *Sac*B gene encodes an enzyme known as levansucrase which is involved in metabolism of levan (Liu et al., 2016; Kumar, Sripada & Poornachandra, 2018). From the experiment, it showed that the mutant GV2260-SacB/R exhibited no growth on LB medium supplement with 5% sucrose, while the wildtype showed unaffected growth in both the presence and absence of 5% sucrose. Apart from

that, Liu et al. (2016) evaluated performance of wild-type strain and mutant strain in transient and stable transformation in plants. For transient transformation experiment, result showed that both strains expressed strong fluorescent signals on *Nicotiana benthamiana* leaves. This indicates that sucrose-sensitive mutation does not alter transient gene delivery. Other than that, researchers also tested the stable plant transformation for mutant strain. Another binary vector, pgRNA-NbWRKY70, was also used. The vector carried a Cas9 gene driven by a strong plant promoter, ubiquitin 10 (UBQ10), and a guide RNA that targets the *WRKY70* gene in *Nicotiana benthamiana* under the *Arabidopsis* U6 promoter. Leaf-disk transformation assays were performed, and leaf disks were briefly washed to promote the persistence of *Agrobacterium*. Liu et al. (2016) showed that the contamination rate caused by mutant GV2260-SacB/R reduced significantly from >80 % in the wildtype to about 13.0% and 26.9% in 5% and 3% sucrose, respectively. The study concluded that the GV2260-SacB/R mutant can be easily inhibited by sucrose in *N. benthamiana* tissue culture medium. In addition, PCR and sequencing results confirmed the presence of the Cas9 transgene and successful introduction of mutations in the target *WRKY70* locus in regenerated plants (Liu et al., 2016). This study demonstrated that GV2260-SacB/R maintains full transformation competence for both transient and stable plant transformations (Liu et al., 2016). Meanwhile, with the incorporation of the *sac*B-*sac*R gene, the problem of *Agrobacterium* overgrowth was resolved. CRISPR editing can also be applied to plants regenerated from GV2260-SacB/R infections (Liu et al., 2016).

2.6 An improvised version of ternary vector system and development of *thy*A-deficient auxotrophic strains for enhanced *Agrobacterium*-mediated plant transformation

In 2024, a study conducted by Aliu et al. has successfully developed a thymidine auxotrophic *Agrobacterium* strain that could resolve the problem of overgrowth in post transformation. The thymidine-dependent *Agrobacterium* strains (EHA101, EHA105, EHA105D, and LBA4404) were generated. Two types of approaches were used to generate the auxotrophic strains: allelic exchange method, and CRISPR-based INTEGRATE mutagenesis. For allelic exchange mutagenesis, a non-functional *thy*A allele was cloned into a suicide vector to replace the native gene via homologous recombination. The resulting sequence after homologous recombination is scar-free, and no selection markers are required (Lazarus et al., 2019; Aliu et al. 2024). In contrast, the CRISPR-INTEGRATE system created thymidine-dependent auxotrophs through the insertion of *mCherry* marker into the coding region of *thy*A. The study by Aliu et al. (2024) showed that both approaches successfully yielded thymidine-dependent auxotrophs. Apart from that, auxotrophic strains constantly showed a lower growth rate compared to their prototrophic counterparts despite thymidine was supplemented. Additionally, thymidine auxotrophic strains exhibit reduced growth in the absence of thymidine when co-cultured with plants. This indicates that auxotrophic strains are safe to be used in plant transformation and higher plant transformation rate can be achieved (Aliu et al. 2024; Goralogia, Willig & Strauss, 2025).

Moreover, Aliu et al. has also successfully developed an improvised version of vir helper plasmid, pKL2299A from pKL2299. An additional *vir*A gene was added, which enables *vir*G and other vir genes to be activated that facilitate T-DNA transfer. The experimental result has shown that pKL2299A gives approximately 1.3 to 1.5 times higher transformation rate compared to pKL2299. In addition, pKL2299A, which has all the essential *vir* genes for plant transformation, can be used not only in *Agrobacterium* but also in other bacterial strains for gene delivery.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental design

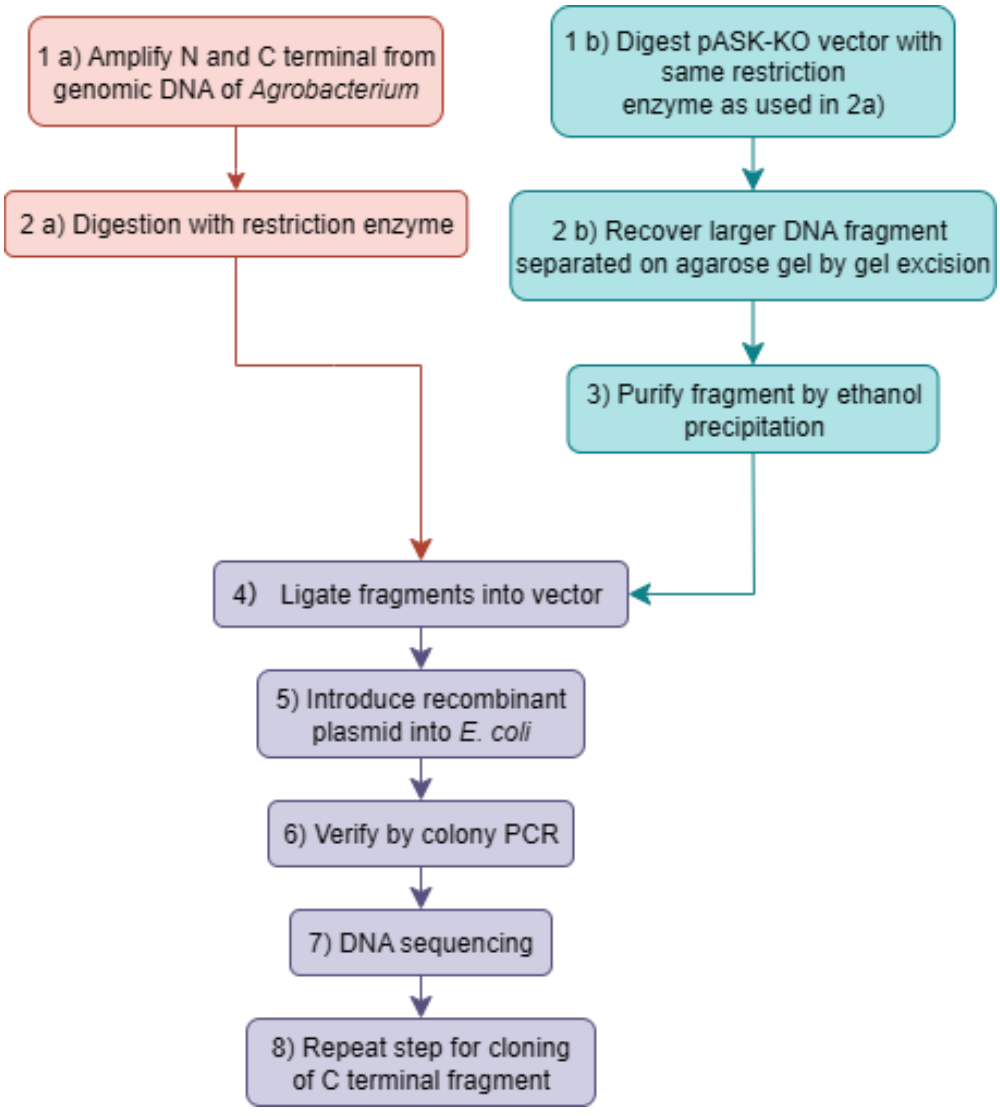


Figure 3.1 Flowchart of overall experimental design

3.2 Preparation of medium, buffer solutions, and competent cells

All chemical reagents, apparatus, equipment, and instruments used throughout the experiment were provided by the laboratory of Department of Biological Science, Faculty of Science, University Tunku Abdul Rahman (UTAR) and my supervisor Prof. Wong Hann Ling. The information for the mentioned items is listed in Appendix G and H.

3.2.1 Preparation of 2X YT culture medium

For 80 mL of 2X YT broth medium, 0.8 g of yeast extract was weighted using analytical balance, followed by 1.28 g of tryptone, and 0.4 g of sodium chloride. The components were mixed in 60 mL of distilled water, and then topped up to 80 mL. Once the solution was well mixed, it was sent for autoclaving.

To prepare agar, the above procedure was repeated, along with 1.2 g of agar powder. After the agar solution was autoclaved, it was cooled to about 50°C before adding antibiotics. To 80 mL of the agar solution, 8 µL of 100 mg/mL streptomycin or 8 µL of 50 mg/mL kanamycin was added. The solution was swirled gently to allow thorough mixing. The agar solution was then poured equally and gently into sterile agar plates in laminar airflow under aseptic conditions. The agar was allowed to solidify in laminar airflow for 30 min before sealing the lid.

3.2.2 Preparation of 5X TBE buffer

A 0.5 M EDTA solution (20 mL) was prepared and used to prepare 500 mL of 5X TBE buffer. First, 3.722 g of EDTA disodium salt was weighed and dissolved in 16 mL of distilled water. The EDTA disodium salt was dissolved by adjusting the pH to 8.0 with 10 M NaOH, drop by drop. The mixing was aided by low heating on a hot plate stirrer. As the solution became clear, it was topped up with distilled water to a volume of 20 mL. On the other hand, 27 g of Tris base and 13.75 g of boric acid were used. Subsequently, 10 mL of 0.5 M EDTA was added to dissolve the Tris base and boric acid. The solution was slowly mixed and topped up with distilled water to 500 mL before autoclaving. Finally, the 500 mL of 5 X TBE buffer was diluted to 0.5 X by adding 4500 mL of sterile distilled water before use.

3.2.3 Preparation of heat shock reagent

To prepare 80 mL of the heat shock reagent, 1.176 g of calcium chloride (142.02 g/mol) was weighed. Then, 12 mL of glycerol and 68 mL of distilled water were used to dissolve calcium chloride. Finally, the mixture was autoclaved.

3.2.4 Preparation of competent cells and heat shock transformation

The *E. coli* TOP 10 was grown in fresh agar plate culture in prior for one overnight. Subsequently, a single colony of *E. coli* was cultured in 3 mL of autoclaved broth culture supplemented with 3 µL of 100 mg/mL streptomycin. The culture was incubated overnight in a shaking incubator at 37°C and 220 rpm. The next day, 200 µL of the overnight culture was withdrawn and transferred to 20 mL of fresh and sterile broth in a 250 mL conical flask. Broth culture was incubated in a shaking incubator at 37°C and 220 rpm for 4 h. The cell culture was harvested by transferring it to a pre-chilled 50 mL centrifuge tube. The cell culture was centrifuged at 3000 xg, for 10 minutes, at 4°C. The supernatant was then discarded. In the next step, the cells were kept on ice. To the pellet, 300 µL of ice-cold heat shock reagent was added and the cell culture was incubated on ice for 20 min. Then, the cell culture was aliquoted into a sterile 1.5 mL microcentrifuge tube with about 50 µL per microcentrifuge tube. During heat shock transformation, 10 µL of the recombinant plasmid was added to each microcentrifuge tube containing competent cells. The cells were then incubated on ice for 15 minutes. After that, all the microcentrifuge tubes were swiftly transferred to a 42°C water bath for 1 min. Followed by that, all microcentrifuge tubes were immediately incubated on ice for 5 minutes. Subsequently, 250 µL of 2X YT broth was added to each microcentrifuge tube for cell recovery. The cell cultures were incubated again at 37°C in a shaking incubator at 220 rpm for 1 h. Finally, 100 µL of recovered transformed cells were withdrawn and plated on agar plates supplemented with 50 µg/mL kanamycin. The spread plate method was used to evenly plate cells on an agar

plate using a glass spreader. Finally, the agar plates were incubated overnight at 37 °C.

3.3 Culture of *Agrobacterium* in preparation for genomic DNA extraction

A. tumefaciens was cultured in fresh 2X YT broth, as described in Section 3.2.1. A single loop of *Agrobacterium* was inoculated into 5 mL of broth culture in a sterile 50 mL centrifuge tube. The culture was incubated in a shaking incubator at 220 rpm for overnight at 37 °C.

3.4 Extraction of genomic DNA from *Agrobacterium tumefaciens* C58C1

Genomic DNA was extracted using the RBC Bioscience® Genomic DNA Extraction Kit. The overnight *Agrobacterium* culture was transferred to sterile 1.5 mL microcentrifuge tubes and centrifuged for 1 min at 10,000 × g. The supernatant was discarded. Then, 200 µL of GT Buffer was added to the tube and pipetted to resuspend the cell pellet. The cell mixture was then incubated at room temperature for 5 min. Subsequently, 200 µL GB Buffer was added to the sample and vortexed for 5 s to mix the sample. The samples were then incubated at 70°C for 10 min until the sample lysate became clear. During the incubation, the tubes were inverted every 3 min. The elution buffer was then preheated to 70°C. After the incubation, 5 µL of RNase A was added and vortexed. After that, 200 µL of absolute ethanol was added and vortexed for 10 s. A GD Column was positioned on top of a 2 mL collection tube. All contents from the microcentrifuge tube were added to the GD Column, and followed by centrifugation at 10,000 × g for 2 min. The flow-through was discarded, then the

GD column was placed back on top of a 2 mL collection tube. Next, 400 µL of W1 Buffer was added to the GD column and centrifuged for 30 s at 10,000 × g. The flow-through was discarded, and the GD column was placed back in the 2 mL collection tube. Following that, 600 µL of wash buffer was added to the GD column and centrifuged for 30 s at 10,000 × g. After that, the flow-through was discarded, and the column was centrifuged for 3 minutes at full speed.

Following this, the GD Column was transferred to a sterile 1.5 mL microcentrifuge tube. Furthermore, 30 µL of preheated elution buffer was added to the center of the column matrix, and the column allowed to stand for 2 min. DNA was purified at the end of the procedure by centrifuging the tube at 10,000 × g for 30s.

3.5 PCR amplification of N-*rec*A

PCR amplification was conducted to amplify the N-and C terminus of the *rec* A gene. Amplification of the N-and C terminus fragments was performed sequentially. In the first half of the experiment, the N-terminus fragment was PCR-amplified using the primers F-BamHI-recA-N43 and RrecA-N484. The forward primer included a *Bam*HI restriction site that allowed restriction by *Bam*HI in the downstream digestion reaction. The amplified N-terminus fragment was verified by agarose gel electrophoresis. The N-terminus fragment had an expected band size of 450 bp. Table 3.1, 3.2, and 3.3 below shows the

primer sets used for N terminus fragment amplification, PCR components, and thermocycling conditions.

Table 3.1 Primer used for amplification of N terminus fragment

Primer	Sequence (5'-…-3')
F-BamHI-recA-N43	GTGGATCCaacgaaagcagaacaaactactt
R-recA-N484	agatgccgcccttcttctg

*Underlined sequence is the restriction sites for *Bam*HI enzyme

Table 3.2 Components for PCR master mix and the volumes prepared

Component	Volume for single reaction (µL)	Volume for 2 reactions (µL)
Vazyme 2X Taq Master Mix	25	50
Forward primer (10 µM)	2.5	5
Reverse primer (10 µM)	2.5	5
Agrobacterium genomic DNA	2	4
Sterile distilled water	17.7	35.4
Total volume	50	100

Table 3.3 Thermocycling conditions for PCR amplification of N-*rec*A

Step	Temperature (°C)	Time (seconds)	Cycle
Initial denaturation	98	30	1
Denaturation	98	10	35
Annealing	53	30	35
Extension	72	15	35
Final extension	72	120	1
Hold	4	∞	-

3.6 Agarose gel electrophoresis of PCR amplified N-*rec*A

The PCR-amplified N terminus fragment was verified using agarose gel electrophoresis. A 2% agarose gel was prepared by dissolving 0.4 g of agarose powder in 20 mL of 0.5 X of TBE buffer. The 2% agarose gel solution was heated to 90°C until the solution became clear. As the solution cooled to about 50°C, the pre-stain loading dye was added and mixed thoroughly. The gel was cast using a comb. When the gel solidified, it was submerged into 0.5 X TBE buffer in a gel tank. Then, the PCR-amplified sample was loaded into the wells on an agarose gel. Gel electrophoresis was performed at 90 V for 45 min and then visualized using a gel imaging system.

3.7 Purification of PCR-amplified N-*rec*A

Purification of PCR-amplified N-*rec*A was performed using a Macherey Nagel NucleoSpin® gel kit. Two volume of buffer NT1 were mixed with one volume of the sample. A NucleoSpin® gel and a PCR clean-up column were placed in a collection tube. During DNA binding, 700 µL of sample was loaded onto the column. The tube was then centrifuged at 11,000 × g for 30 s. The flow-through was discarded, and the column was returned to the collection tube.

In addition, 700 µL Buffer NT3 was added to the NucleoSpin® gel and PCR Clean up column, followed by centrifugation at 11,000 × g for 30 s. The flow-through was discarded, and the column was returned to the collection tube. Centrifugation was repeated for another 1 min to thoroughly remove Buffer NT3. Finally, the NucleoSpin® gel and PCR clean-up column were transferred to a

new 1.5 mL microcentrifuge tube. Next, 25 µL of Buffer NE was added to elute DNA, and the sample was incubated at room temperature for 1 min. The mixture was then centrifuged for 1 min at 11,000 g.

3.8 Culture of *E. coli* TOP 10 pASK-KO

E. coli TOP 10 pASK-KO was cultured in 5 mL sterile broth supplemented with 5 µL of 100 mg/mL streptomycin antibiotic in 50 mL centrifuge tube. A single loop of *E. coli* was inoculated into the broth and allowed to grow overnight at 37°C in 220 rpm shaking incubator overnight.

3.9 Plasmid extraction from *E. coli* culture

Plasmid extraction from the *E. coli* culture was performed using the PrimeWay Plasmid DNA Extraction Kit (1st Base). First, the *E. coli* culture was pelleted by centrifugation at 11,000 × g for 1 min and the supernatant was discarded. Subsequently, 250 µL of pD1 buffer (with RNase A) was added to the cell suspension and resuspended. The cell suspension was then transferred to a new 1.5 mL microcentrifuge tube. Next, 250 µL pDL2 buffer was added and mixed thoroughly. The cell suspension was treated for 5 min. Next, 350 µL of pDL3 buffer was added, and the tube was inverted and flipped 10 times, which was followed by centrifugation at 18,000 × g for 10 min. The supernatant was carefully transferred to a PrimeWay Plasmid Column and centrifuged for 30 s at 11,000 g. Flow-through was discarded. 400 µL Wash Buffer A1 was then added,

followed by centrifugation for 30 s at 11,000 × g. The flow-through was discarded. This was repeated with 700 µL Wash Buffer A2. The cells were centrifuged at 18,000 g for 3 min. The Elution Buffer (50 µL) was added to the center of the column, and the column was allowed to sit at room temperature for 1 min.

3.10 Restriction digestion of N-*rec*A and pASK-KO

The concentration of the extracted plasmid and PCR-amplified N-terminal fragment of *rec*A was measured using a NanoDrop Spectrophotometer. Restriction enzyme restriction digestion was performed, as shown in Table 3.4.

Table 3.4 Enzyme restriction digestion of PCR amplified N-*rec*A and pASK-KO

Component	**Volume (µL)**	
	PCR-amplified N-*rec*A	**pASK-KO**
Insert / Vector	16.0	7.0
10X buffer	2.0	2.5
Sterile distilled water	1.0	14.5
Enzyme *Bam*HI	0.5	0.5
Enzyme *Pst*I	0.5	0.5
Total volume	20.0	25.0

The enzyme restriction digestion reaction was performed in a 37°C water bath for 3 h.

3.11 Gel electrophoresis to verify the restriction digestion of N-*rec*A and pASK-KO

After restriction digestion, the products were subjected to gel electrophoresis on 1.2% agarose. The products were loaded onto an agarose gel and the gel electrophoresis was run at 90 V for 45 min.

3.12 Purification of digested N-*rec*A

Digested N-*rec*A was purified using a kit from Macherey Nagel NucleoSpin® gel, as described previously in subsection 3.7.

3.13 Purification of pASK-KO through gel excision method

After gel electrophoresis, the pASK-KO fragments were recovered using the gel excision method. The entire agarose gel was placed on a UV transilluminator to visualize the position of the band. Bands were carefully excised using a sharp sterile blade. The excised band was further trimmed to remove excess agarose gel surrounding it. The gel was placed into a 1.5 mL microcentrifuge tube, 500 µL of Buffer B. Then, and the gel was melted at 50°C. Once the gel had completely melted, it was purified using a Macherey Nagel NucleoSpin® gel kit, following the procedure described in Section 3.7, starting from the DNA binding step.

3.14 Ligation of N-*rec*A fragment with pASK-KO vector to form recombinant plasmid

The final concentrations of N-*rec*A fragment and pASK-KO were measured using a NanoDrop Spectrophotometer. As such, the number of inserts and vectors required, as well as their respective volume required, can be calculated.

Table 3.5 Information to calculate the insert: vector ratio for ligation of N-*rec*A and pASK -KO

	Concentration (ng/µL)	Amount required (ng)	Volume required (µL)	Volume obtained after purification (µL)
Insert	90	558.5	6.2	20
Vector	50	1000	20	20

With the given information keyed in into NEBioCalculator,

Insert DNA length = 450 bp

Vector DNA length = 4029 bp

Vector DNA mass = 1000 ng

The largest ratio of insert: vector that could be applied for ligation in this experiment was 5:1, which was 558.5 ng of insert and 1000 ng of vector. The concentrations of the insert and vector obtained after purification were 90 ng/µL and 50 ng/µL, respectively. Therefore, the volume of the insert and vector required for ligation in the ratio of 5:1 would be 6.2 µL of insert and 20 µL of vector.

Table 3.6 Components and the volume used for ligation of N-*rec*A and pASK-KO

Component	Volume (µL)
Insert (N-*rec*A)	6.2
Vector (pASK-KO)	20
T4 DNA Ligase	1.0
10 X Buffer	3.2
Sterile distilled water	1.6
Total volume	32

After all the components were added to a 1.5 mL microcentrifuge tube, it was incubated in a PCR machine at 16°C for overnight. Subsequently, the ligated products were transformed into competent *E. coli* cells using the heat-shock transformation method described in Section 3.2.4.

3.15 Screening for transformed recombinant clone by colony PCR

3.15.1 Vector-specific colony PCR

All transformation plates were screened for transformed recombinant clone pASK-NrecA-repA. Vector-specific primers were used for first-round colony PCR screening. The primer set, components of the master mix, and thermocycling conditions were as follows.

Table 3.7 Vector-specific primers used in the preliminary colony PCR screening of transformed recombinant clone

Primer	Primer name	Sequence (5'…3')
Forward primer	F-Kan11-Seq	cctcgagcaagacgtttcc
Reverse primer	R-Pst1-NASD	CACCTGCAGGcgacttcatctgccggaaaac

Table 3.8 Components for vector-specific colony PCR master mix and the volumes prepared

Component	Volume for single reaction (µL)	*Volume for 60 reactions (µL)
10X Homemade Taq Buffer	1.0	65
25 mM $MgCl_2$	0.8	52
2.5 mM dNTP	0.8	52
Forward primer (10 µM)	0.4	26
Reverse primer (10 µM)	0.4	26
Sterile distilled water	6.2	403
DNA polymerase	0.4	26
DNA	-	-
Total volume (µL)	10	650

* The volume prepared for 60 reactions was in excess of 5 µL to account for errors in pipetting.

Table 3.9 Thermocycling conditions for vector-specific colony PCR in screening putative recombinant clone

Step	Temperature (°C)	Time (seconds)	Cycle
Initial denaturation	95	600	1
Denaturation	95	10	35
Annealing	50	30	35
Extension	72	60	35
Final extension	72	120	1
Hold	4	∞	-

After the master mix was prepared, DNA was introduced into each PCR tube by randomly picking colonies using one end of a sterile toothpick from transformation plates. The colonies were streaked onto replica plates every time they were introduced into the PCR tubes of the reaction mix. All picked colonies were assigned numbers that corresponded to the numbering on the replica plate to ease the tracking process of the specific colonies in search. Then, all reaction tubes were placed in a PCR machine with the thermocycling set according to the values in Table 3.9 to undergo polymerase chain reaction. Colony PCR was repeated until the desired clones were identified.

3.15.2 Verification of recombinant clones, pASK-NrecA-repA by gel electrophoresis

The expected band size resulting from the vector-specific amplification was 452 bp. The putative recombinant clones were run on 2% agarose gel electrophoresis at 90 V for 45 minutes.

3.15.3 Orientation-specific colony PCR

The second round of verification using orientation-specific primers produced an expected amplicon of approximately 3027 bp. The orientation-specific primers, components of master mix, and thermocycling conditions were shown in table 3.10, 3.11, and 3.12, respectively.

Table 3.10 Primer used for colony PCR in screening transformed pASK-NrecA-repA

Primer	Primer name	Sequence (5'…3')
Forward primer	F-BamHI-recA-N43	GTGGATCCaacgaaagcagaacaaactactt
Reverse primer	R-Ori108-Seq	gtttcgccacctctgacttg

*Underlined sequence is the restriction sites for *Bam*HI enzyme

Table 3.11 Components for colony PCR master mix and the volumes prepared

Component	Volume for single reaction (µL)	Volume for 8 reactions (µL)
Vazyme 2X *Taq* Master Mix	5	40
Forward primer (10 µM)	0.5	4
Reverse primer (10 µM)	0.5	4
Sterile distilled water	3	24
DNA	1	8
Total volume (µL)	10	80

Table 3.12 Thermocycling conditions for orientation-specific colony PCR in screening putative recombinant clone

Step	Temperature (°C)	Time (seconds)	Cycle
Initial denaturation	98	30	1
Denaturation	98	10	35
Annealing	53	30	35
Extension	72	120	35
Final extension	72	120	1
Hold	4	∞	-

3.15.4 Verification of recombinant clones by gel electrophoresis

The putative clones were electrophoresed on a 1.2% gel at 90 V for 45 min. Clones that produced an amplicon size of about 3027 bp were subjected to the next verification step by restriction digestion.

3.16 Enzyme restriction digestion of putative recombinant clone, pASK-NrecA-repA

The putative recombinant clone, clone 99, was identified and verified by enzyme restriction digestion to confirm the presence of N-*rec*A. Enzyme *Eco*RV was used to perform a single cut in the recombinant plasmid. The *Eco*RV restriction site is unique in the recombinant plasmid and is only present within N-*rec*A. Restriction digestion was performed, as shown in Table 3.13.

Table 3.13 Components used for restriction digestion of pASK-NrecA-repA

Component	pASK-KO digestion Volume (µL)	Putative recombinant clone (clone 99) digestion Volume (µL)
DNA	5	3
Buffer	1	1
Sterile distilled water	3.5	5.5
*Eco*RV	0.5	0.5
Total	10	10

3.17 Gel electrophoresis to confirm success of restriction digestion on pASK-NrecA-repA

Gel electrophoresis using 1.2 % agarose gel was performed to verify the success of restriction digestion. A negative control was included using an unmodified intact pASK-KO plasmid and a digested version of pASK-KO. The products of undigested pASK-KO, digested pASK-KO, undigested clone 99, and digested clone 99 were loaded in volumes of 3 µL, 5 µL, 1 µL, and 3 µL, respectively. The gel was run at 90 V for 45 minutes. Besides, 1kb DNA ladder was loaded on both sides of the gel next to the samples. Clone 99 was outsourced for DNA sequencing.

3.18 Storage of verified pASK-NrecA-repA for inventory

The DNA sequence-verified pASK-NrecA-repA clone was stored for future use. The clone 99 was inoculated into 5 mL 2X YT broth supplemented with 5 µL of 50 µg/mL kanamycin. The culture was grown for overnight in shaking incubator at 37°C, 220 rpm. The following day, the bacterial clone was stored in a cryopreservation tube containing 500 µL of 50 % glycerol and 500 µL of overnight bacterial culture. Finally, the glycerol stock was stored at -80°C freezer.

3.19 Extraction of recombinant plasmid pASK-NrecA-repA

The recombinant plasmid pASK-NrecA-repA was extracted using PrimeWay Plasmid DNA Extraction Kit (1st Base) using the same protocol described in Section 3.9. The extracted pASK-NrecA-repA was used as a starting vector to clone the C terminus fragment of *rec*A in the second part of the experiment.

3.20 PCR amplification of C-*rec*A

PCR was performed to amplify the C-*rec*A fragment. Tables 3.14, 3.15, and 3.16 below shows the primer sets used for C-terminus fragment amplification, PCR component, and thermocycling conditions.

Table 3.14 Primer used for amplification of C terminus fragment

Primer	Sequence (5'-…-3')
F-AscI-recA-C646	aatttGTGGCGCGCCgattcggttgcggccttgac
R-SphI-recA-C1209	GTGCATGCggcaatcagaccggcattc

*Underlined sequence is the restriction sites for *Asc*I enzyme

Table 3.15 Components for PCR master mix and the volumes prepared for C-*rec*A amplification

Component	Volume for single reaction (µL)	Volume for 2 reactions (µL)
Vazyme 2X Taq Master Mix	25	50
Forward primer (10 µM)	2.5	5
Reverse primer (10 µM)	2.5	5
Agrobacterium genomic DNA	5	10
Sterile distilled water	15	30
Total volume	50	100

Table 3.16 Thermocycling conditions for C-*rec*A PCR amplification

Step	Temperature (°C)	Time (seconds)	Cycle
Initial denaturation	98	30	1
Denaturation	98	10	35
Annealing	62	30	35
Extension	72	20	35
Final extension	72	120	1
Hold	4	∞	-

3.21 Agarose gel electrophoresis of PCR amplified C-*rec*A

The expected amplicon size of C-*rec*A was 587 bp. Therefore, 2% agarose gel was used to run the sample at 90 V for 45 min.

3.22 Purification of PCR-amplified C-*rec*A

Purification of PCR-amplified C-*rec*A was done using a PCR Clean-Up and Gel Extraction kit (GeneDireX®). First, 500 µL of Buffer B was added to the PCR product and mixed by vortexing. The mixture was incubated at 60°C for 10 min. The tube was inverted every 2-3 minutes. A PG Column was placed in a collection tube, and the supernatant was applied to the column. The column was then centrifuged at 14,000 × g for 30 s. Subsequently, flow-through was discarded. During the washing step, 400 µL of Buffer W1 was added to the PG Column and centrifuged at 14,000 × g for 30 s. The flow-through was discarded, and the PG Column was returned to the collection tube. This step was repeated for another round by adding 600 µL of Buffer W2. Finally, the residual buffer was removed by centrifugation at 14,000 × g for 2 min. In the elution step, the PG Column was transferred to a sterile 1,5 mL microcentrifuge tube. Then, 50 µL of Buffer BE was added to the center of the PG Column and allowed to stand for 2 min before centrifugation at 14,000 × g for 2 min.

3.23 Restriction digestion of C-*rec*A and pASK-NrecA-repA

Restriction enzyme digestion of C-*rec*A and pASK-NrecA-repA was performed, as shown in Table 3.17. The enzyme restriction digestion reaction was performed in a 37°C water bath for overnight.

Table 3.17 Enzyme restriction digestion of PCR-amplified C-*rec*A and pASK-NrecA-repA

Component	**Volume (µL)**	
	PCR-amplified C-*rec*A	**pASK-NrecA-repA**
Insert / Vector	20.0	10.0
10X buffer	2.5	1.5
Sterile distilled water	1.5	2.5
Enzyme *Asc*I	0.5	0.5
Enzyme *Sph*I	0.5	0.5
Total volume	25.0	15.0

3.24 Gel electrophoresis to verify the restriction digestion of C-*rec*A and pASK-NrecA-repA

Gel electrophoresis was performed on a 1.2% agarose gel at 90 V for 45 min.

3.25 Purification of restriction enzyme-digested C-*rec*A

Purification of restriction enzyme-digested C-*rec*A is described in Section 3.22.

3.26 Purification of pASK-NrecA-repA through gel excision method

The purification of the vector pASK-NrecA-repA was performed using the gel excision method, which was previously outlined in Section 3.13.

3.27 Ligation of C-*rec*A with pASK-NrecA-repA vector to form recombinant plasmid

The final concentration of the C-*rec*A fragment and pASK-KO obtained was measured, and the amount of insert and vector required, as well as their respective volume required, were calculated prior to the ligation reaction.

Table 3.18 Data used to calculate the insert: vector ratio for ligation

	Concentration (ng/µL)	**Amount required (ng)**	**Volume required (µL)**	**Volume obtained after purification (µL)**
Insert	44	660	15	15
Vector	25.0	500	20	20

With the given information keyed in into NEBioCalculator,

Insert DNA length = 587 bp

Vector DNA length = 3998 bp

Vector DNA mass = 56 ng

The applicable insert: vector ratio was 7:1, and the amount of inserted DNA mass needed was 513.9 ng. Because the amount of C-*rec*A (15 µL) was more than the required amount, all 15 µL could be utilized. Table 3.19 shows the components added to the ligation tube.

Table 3.19 Components and the volume used for ligation of C-*rec*A with pASK-NrecA-repA

Component	Volume (µL)
Insert (C-*rec*A)	15.0
Vector (pASK-NrecA-repA)	20.0
T4 DNA Ligase	1.0
10 X Buffer	4.5
Sterile distilled water	4.5
Total volume	45.0

Ligation tubes were incubated overnight in a PCR machine at 16 °C for 1 h. The ligated products were then transformed into competent *E. coli* cells using the heat shock transformation method described in Section 3.2.4.

3.28 Screening for transformed recombinant clones using gene-specific colony PCR.

All transformation plates were screened for the transformed recombinant clone pASK-NrecA-repA-CrecA. The primer set, components of master mix, and thermocycling conditions are shown in Tables 3.20, 3.21, and 3.22.

Table 3.20 Gene-specific primers used for colony PCR screening of transformed recombinant clone

Primer	Primer name	Sequence (5'…3')
Forward primer	F-AscI-recA-C646	aatttGTGGCGCGCCgattcggttgcggccttgac
Reverse primer	R-SphI-recA-C1209	GTGCATGCggcaatcagaccggcattc

*Underlined sequence is the restriction sites for *Asc*I and *Sph*I enzyme

Table 3.21 Master mix for gene-specific colony PCR and volume preparation.

Component	Volume for single reaction (µL)	Volume for 140 reactions (µL)
10X Homemade Taq Buffer	1.0	145
25 mM $MgCl_2$	0.8	116
2.5 mM dNTP	0.8	116
Forward primer (10 µM)	0.4	58
Reverse primer (10 µM)	0.4	58
Sterile distilled water	6.2	899
DNA polymerase	0.4	58
DNA	-	-
Total volume (µL)	10	1450

* The volume prepared for 140 reactions was in excess of 5 µL to account for errors in pipetting.

Table 3.22 Thermocycling conditions for gene-specific colony PCR in screening putative recombinant clone

Step	Temperature (°C)	Time (seconds)	Cycle
Initial denaturation	95	600	1
Denaturation	95	10	35
Annealing	50	30	35
Extension	72	60	35
Final extension	72	120	1
Hold	4	∞	-

Once the master mix was prepared, DNA was introduced by tooth-picking colonies from the transformation plates onto replica plates and then added to each PCR tube. The details of colony PCR are presented in Section 3.15.1.

3.29 Verification of recombinant clones, pASK-NrecA-repA-CrecA by gel electrophoresis

The sample was subjected to 2% gel electrophoresis, as it had an expected band size of 580 bp. The sample was operated at 90 V for 45 min.

3.30 Enzyme restriction digestion of putative recombinant clone, pASK-NrecA-repA-CrecA

The putative recombinant clones (123, 128, and 129) were further verified by *Eco*RV restriction digestion to confirm the knock-in of C-*rec*A.

Table 3.23 Components used for restriction digestion of pASK-recA-repA-CrecA

	pASK-KO digestion	**Putative recombinant clone (clone 129) digestion**
Component		**Volume (µL)**
DNA	5	5
Buffer	1	1
Sterile distilled water	3.5	3.5
*Eco*RV	0.5	0.5
Total	10	10

3.31 Gel electrophoresis to confirm success of restriction digestion on pASK-NrecA-repA-CrecA

The products of the undigested pASK-KO, digested pASK-KO, undigested clone 129, and digested clone 129 were loaded in volumes of 1, 3.5, 1, and 4 µL, respectively. Gel electrophoresis was performed using a 1.2% agarose gel, and the sample was run at 90V for 45 min. Moreover, 100 bp and 1 kb ladders were loaded on the sides of the gel next to the samples. Finally, verified clone 129 was sent for DNA sequencing.

3.32 Storage of verified pASK-NrecA-repA-CrecA for inventory

Similar to clone 99, sequencing verified that pASK-NrecA-repA-CrecA was stored in a glycerol stock for inventory using the same protocol as described in Section 3.18.

3.33 Extraction of recombinant plasmid pASK-NrecA-repA-CrecA

The recombinant plasmid (clone 129) was extracted using the NucleoSpin ® Plasmid extraction kit. The overnight culture of *E. coli* was centrifuged using a benchtop microcentrifuge for 30 s at 11,000 × g. The supernatant was discarded. Next, 250 µL of Buffer A1 was added to the cell pellet and resuspended by pipetting. In addition, 250 µL of Buffer A2 was added, and the contents were mixed by inverting the tube until the lysate became clear. Additionally, 300 µL of Buffer A3 was added and the tube was mixed by inverting until the blue coloration became clear. The lysate was centrifuged for 5 min at 11,000 × g at room temperature to obtain a clear supernatant. In the following step, a NucleoSpin® Plasmid Column was inserted into a collection tube, and 700 µL of the supernatant from the previous step was added. The NucleoSpin® Plasmid Column was spun at 11,000 g for 1 minute, and the flow-through was discarded. The NucleoSpin® Plasmid Column was placed back into the collection tube. This was done repeatedly until all of the lysates had been used. During the wash step, 600 µL of Buffer A4 was added, and the NucleoSpin® Plasmid Column was spun at 11,000 × g for 1 min. The flow-through from the NucleoSpin® Plasmid Column was discarded and the NucleoSpin® Plasmid Column was placed back into the emptied collection tube. The NucleoSpin® Plasmid Column

was spun at 11,000 × g for 2 minutes to remove residual ethanol from the wash buffer.

The NucleoSpin® Plasmid Column was placed into a sterile 1.5 mL microcentrifuge tube, and 50 µL of Buffer AE was added. The mixture was incubated at room temperature for 1 min and centrifuged for 1 min at 11,000 × g.

CHAPTER 4

RESULT

4.1 PCR amplification of N-*rec*A

The N terminal of *rec*A gene was isolated from *Agrobacterium* by PCR amplification. Figure 4.1 shows the gel electrophoresis result. The amplified N-*rec*A had the expected size of about 450 bp. Lanes 4 and 5: *Agrobacterium* genomic DNA were included for comparison.

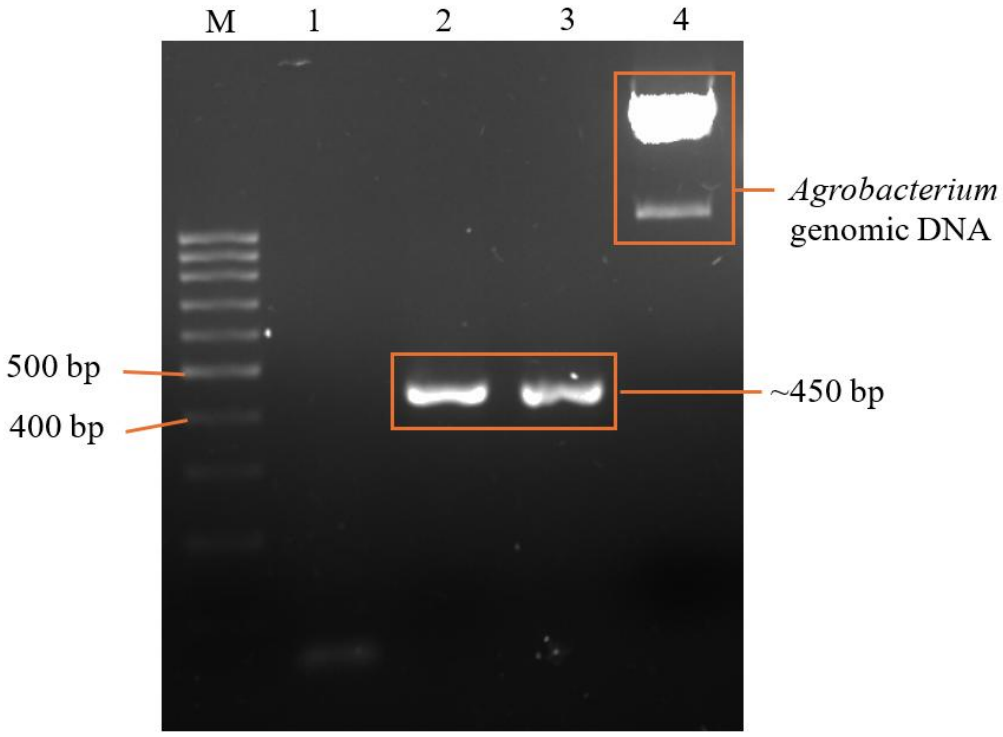


Figure 4.1: **PCR amplification of the N-terminus of *rec*A gene.**
Lane M: 100 bp DNA ladder
Lane 1: Negative control without DNA template
Lane 2-3: Amplified N-*rec*A
Lane 4: *Agrobacterium* genomic DNA

4.2 Restriction digestion of N-*rec*A and pASK-KO with *Bam*HI and *Pst*I

Enzyme restriction digestion was carried out using *Bam*HI and *Pst*I to create complementary ligation ends on N-*rec*A and the vector pASK-KO (see Appendix F). The digested pASK-KO showed two distinct bands with a larger fragment coming from the main vector backbone and the smaller fragment to be excluded from the main backbone to create a flanking site used in downstream cloning. In addition, digested N-*rec*A had a band size of 412 bp. The gel electrophoresis result was shown in Figure 4.2.

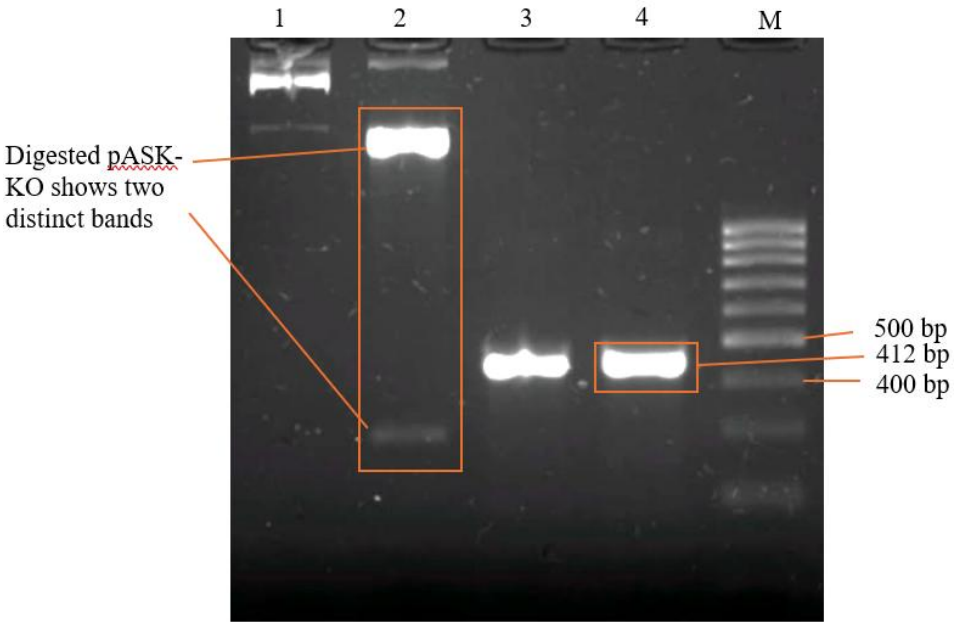


Figure 4.2: Gel electrophoresis of *Bam*HI and *Pst*I double-digested N-*rec*A and pASK-KO on a 1.2% (w/v) agarose gel.
Lane 1: Undigested pASK-KO.
Lane 2: *Bam*HI and *Pst*I double-digested pASK-KO
Lane 3: Undigested N-*rec*A
Lane 4: *Bam*HI and *Pst*I double-digested N-*rec*A gene
Lane M: 100 bp DNA ladder

4.3 Transformation of ligated pASK-NrecA-repA into *E. coli* TOP10 cells.

Figure 4.3 shows the transformant colonies obtained from the transformation of *E. coli* TOP10 cells with ligated pASK-NrecA-repA. Transformed cells were plated on 2X YT plates containing kanamycin.

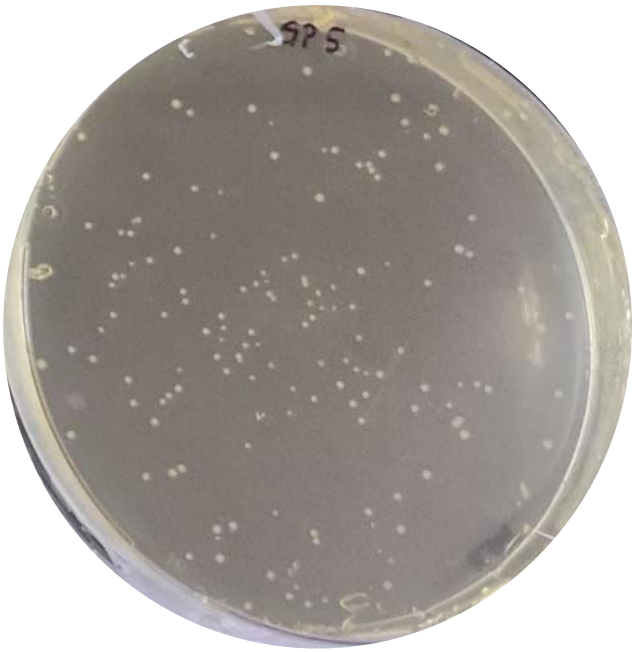


Figure 4.3: Transformation of *E. coli* TOP10 with pASK-NrecA-repA.

4.4 Colony PCR as screening method for recombinant pASK-NrecA-repA

Figure 4.4 illustrates the screening for recombinant clones by picking clones and streaking them on a replica plate supplemented with kanamycin. All clones were labelled with their respective numbers to perform colony PCR.

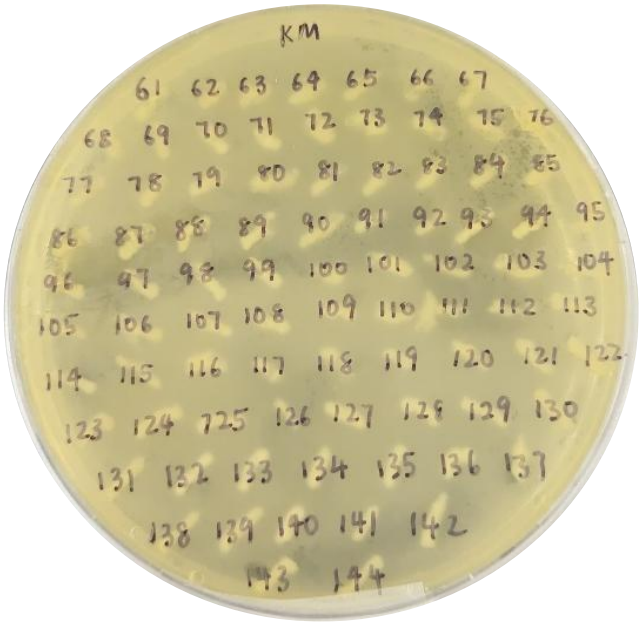


Figure 4.4: Master plate of pASK-NrecA-repA transformants prepared for colony PCR screening.

4.4.1 Preliminary colony PCR screening by using vector-specific primers

The first round of gel electrophoresis, using vector-specific primers, is shown in Figure 4.5. The primers used were F-Kan11-Seq and R-PstI-N. Nonmodified clones produced an amplicon of 452 bp, whereas modified clones did not produce a band. This was due to the disruption by the addition of N-*rec*A at the recognition site of primer R-PstI-NASD, thereby inhibiting amplification by the primer. The gel electrophoresis result of vector specific amplification is shown in Figure 4.5. Clones without bands were selected for further verification using orientation specific primers. As shown in Figure 4.5, clones in lanes 4, 7-12, and 14 were chosen for the second colony PCR screening.

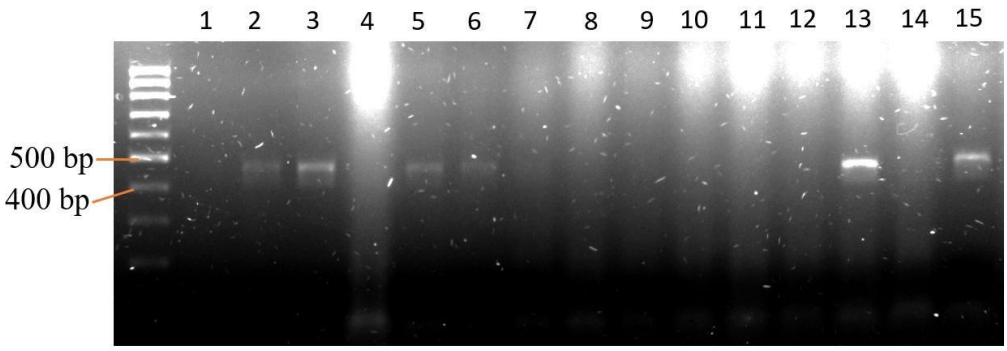


Figure 4.5: Gel electrophoresis of colony PCR for screening recombinant clones using F-Kan11-Seq and R-PstI-NASD primers.
Lane M: 100 bp DNA ladder
Lane 1: Negative control without DNA template
Lane 2: Positive control with an unmodified pASK-KO clone
Lanes 3, 5, 6, 13, 15: Clones excluded from second colony PCR screening
Lanes 4, 7-12, & 14: Clones selected in second colony PCR screening

4.4.2 Colony PCR for verifying the insert orientation of recombinant clones

The clones selected from the first-round colony PCR may have provided clues regarding the success of N-*rec*A cloning. The clones were subjected to orientation-specific amplification to verify the correct orientation of the inserted gene. The gel electrophoresis results are shown in Figure 4.6. A desired clone should produce an amplicon with an expected band size of approximately 3027 bp. In Figure 4.6, only clone 99 in lane 4 (previously in Figure 4.5, lane 8) showed a positive result with a correct band size of 3027 bp.

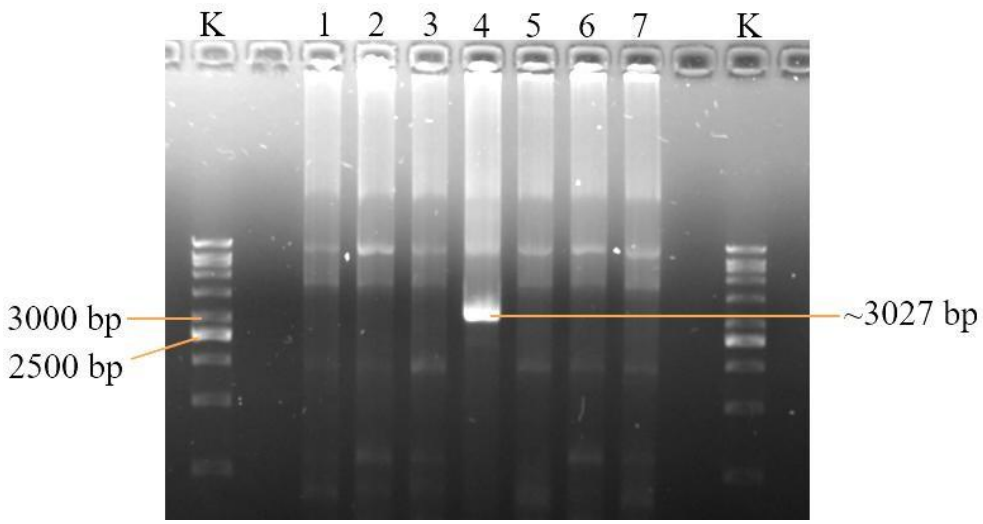


Figure 4.6: Gel electrophoresis of colony PCR for verifying the insert orientation of recombinant clones. Clone 99 in lane 4 showed the expected band size of 3027 bp.
Lane K: 1kb DNA ladder.
Lane 1: Negative control without DNA template
Lane 2: Positive control with an unmodified pASK-KO clone
Lane 3, 5, 6, 7: Clones with negative result
Lane 4: Recombinant clone with N-*rec*A inserted in correct orientation

4.5 Verification of pASK-Nrec-repA clone by restriction digestion

The verified clone 99 was further tested using *Eco*RV restriction digestion. *Eco*RV is expected to make a single cut in the N-*rec*A, which linearises the plasmid to one fragment of ~4630 bp. The undigested pASK-KO, digested pASK-KO, and undigested clone 99 were not cut by *Eco*RV; therefore, no bands were observed in the respective lanes.

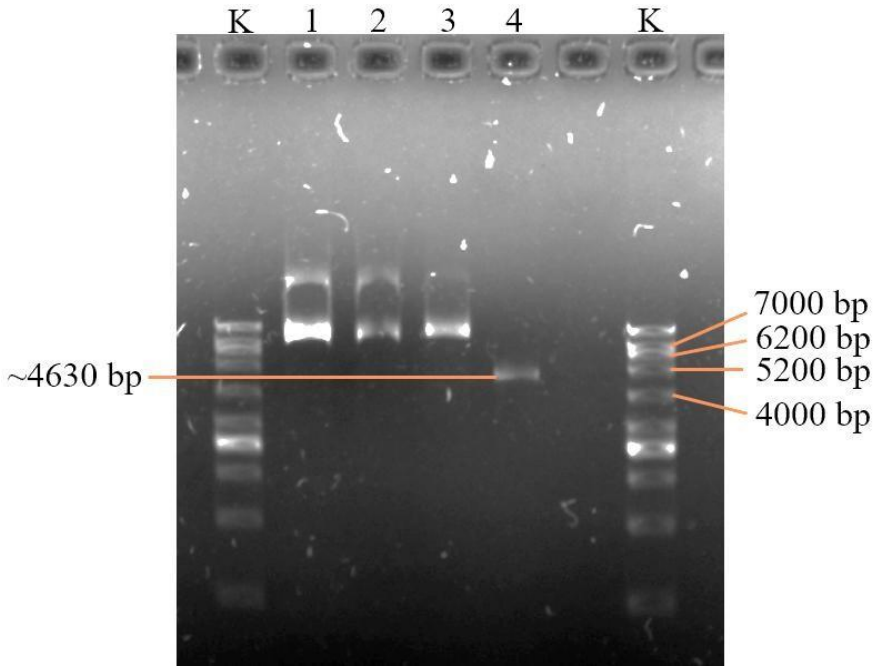


Figure 4.7: Gel electrophoresis of *Eco*RV-digested for recombinant clone 99. A distinct difference in band position was observed between the control unmodified pASK-KO and *Eco*RV-digested clone 99. Lane 4 shows that clone 99 was digested with *Eco*RV and the plasmid was linearized to a fragment of 4630 bp.
Lane K: 1 kb DNA ladder
Lane 1: Undigested pASK-KO
Lane 2: *Eco*RV-digested pASK-KO
Lane 3: Undigested clone 99
Lane 4: *Eco*RV-digested clone 99

4.6 Sequencing pASK-NrecA-repA

The putative recombinant pASK-NrecA-repA clones were outsourced for DNA sequencing using F-Kan11-Seq primer to verify the sequence of the pASK-NrecA-repA insert. The sequencing results were trimmed to a length of 1041 bp before sequence alignment using the BLASTN software and compared with the expected sequence of recombinant plasmid pASK-NrecA-rep. The result is showed in Appendix A. The sequencing results showed 100% identity matches and no gaps. See Appendix A for the sequence alignment.

4.7 PCR amplification of C-*rec*A

The C-*rec*A fragment was PCR-amplified from *Agrobacterium* genomic DNA and had an expected band size of 587 bp (Figure 4.8).

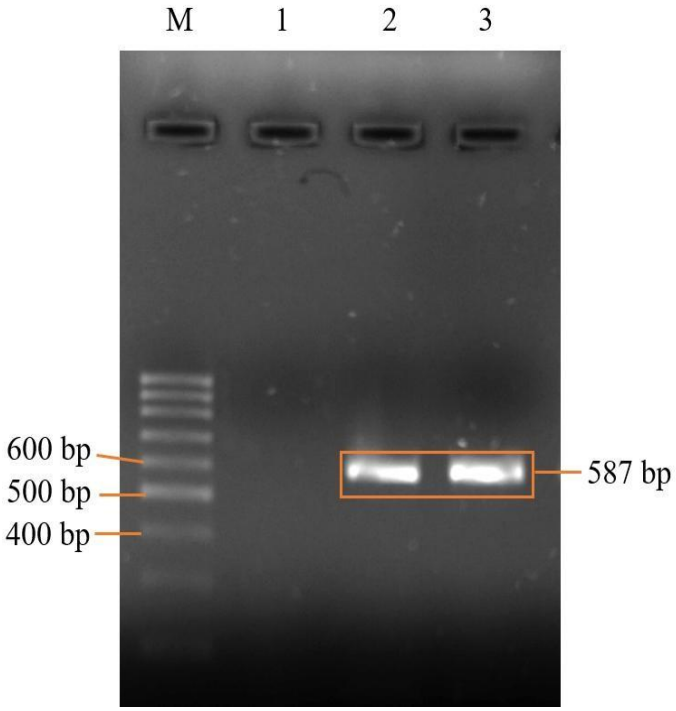


Figure 4.8: Gel electrophoresis of *Asc*I and *Sph*I double-digested C-*rec*A on a 2% (w/v) agarose gel.
Lane M: 100 bp DNA ladder
Lane 1: Negative control without plasmid
Lanes 2 & 3: Amplified C-*rec*A

4.8 Restriction digestion of C-*rec*A and pASK-NrecA-repA with *Asc*I and *Sph*I

Restriction digestion of the recombinant plasmid pASK-NrecA-repA (lane 4) produced a linearized fragment of approximately 4320 bp. In addition, the undigested pASK-NrecA-repA (lane 3) produced two bands, which may reflect the varied conformation of the plasmid. The enzyme-digested and undigested C-*rec*A showed band sizes of approximately 575 bp and 587 bp, respectively. However, the difference in band positions may not be clear due to highly similar band sizes.

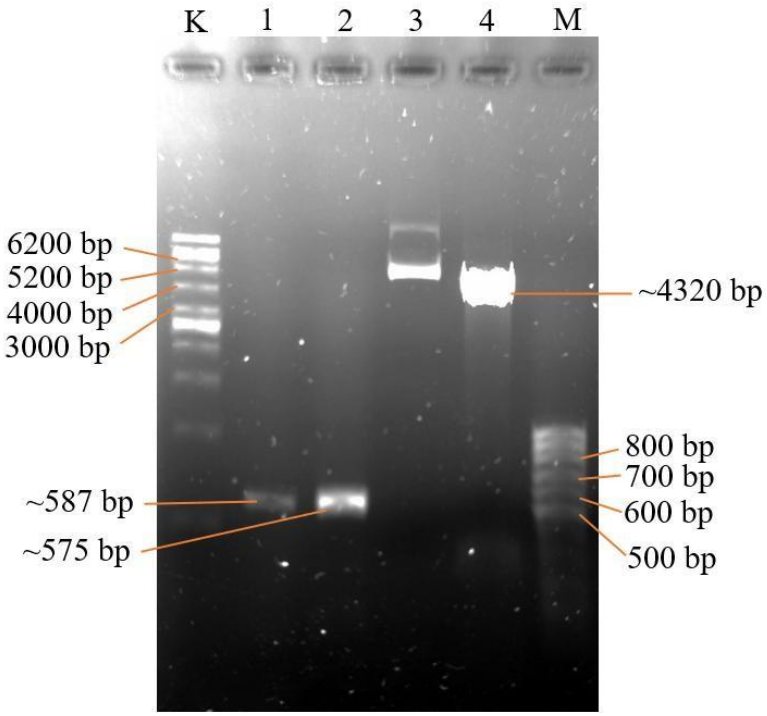


Figure 4.9: Gel electrophoresis of *Asc*I and *Sph*I double-digested recombinant plasmid pASK-NrecA-repA on a 1.2% (w/v) agarose gel.
Lane K: 1 kb DNA ladder
Lane 1: Undigested C-*rec*A
Lane 2: *Asc*I and *Sph*I double-digested C-*rec*A
Lane 3: Undigested pASK-NrecA-repA
Lane 4: *Asc*I and *Sph*I double-digested pASK-NrecA-repA
Lane M:100 bp DNA ladder

4.9 Colony PCR for verifying the insert of recombinant pASK-NrecA-repA

The presence of the insert in recombinant pASK-NrecA-repA was verified using the gene-specific primers F-AscI-recA-C646 and R-SphI-recA-C1209 by colony PCR. The results are shown in Figure 4.10.

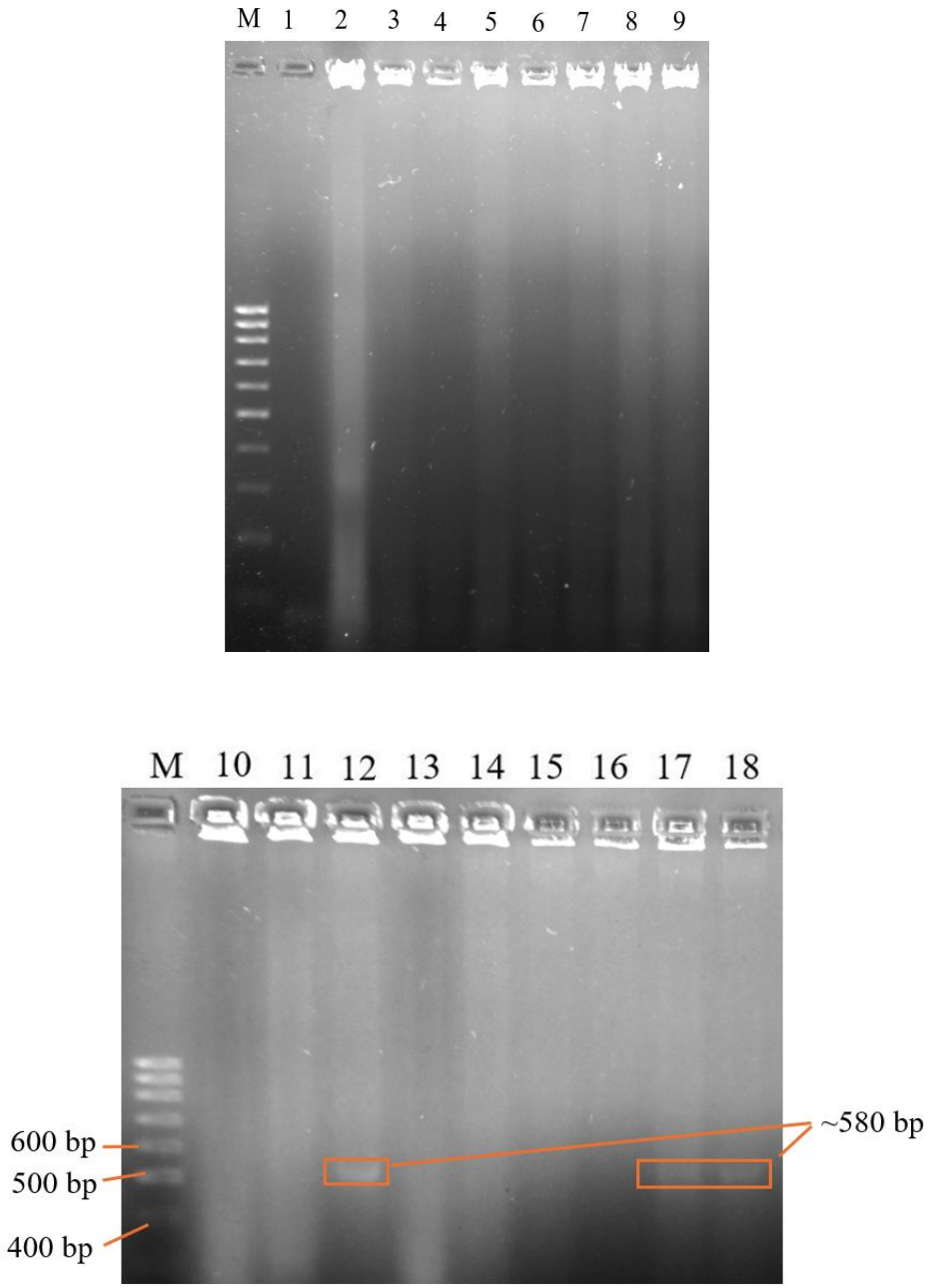


Figure 4.10: Gel electrophoresis of amplification using the gene-specific primers F-AscI-recA-C646 and R-SphI-recA-C1209.

Lane M: 100 bp DNA ladder
Lane 1: Negative control without DNA template
Lane 2-9, 10-11, & 13-16: Clones with negative result
Lanes 12, 17, 18: Recombinant clone showing expected band size

4.10 Verfication of pASK-recA-repA-CrecA clone by restriction digestion

The three putative recombinant pASK-recA-repA-CrecA clones were designated as clones 123, 128, and 129. From these identified clones, clone 129 was selected for further verification through enzyme restriction digestion using *Eco*RV. The gel electrophoresis results are shown in Figure 4.11. Lanes 1-3 shows similar band positions, and pASK-KO has two bands. In addition, the plasmids were not digested using *Eco*RV. Recombinant clone 129 in lane 4 was linearized by *Eco*RV, as the band position revealed the true band size that matched the expected band size of 4895 bp.

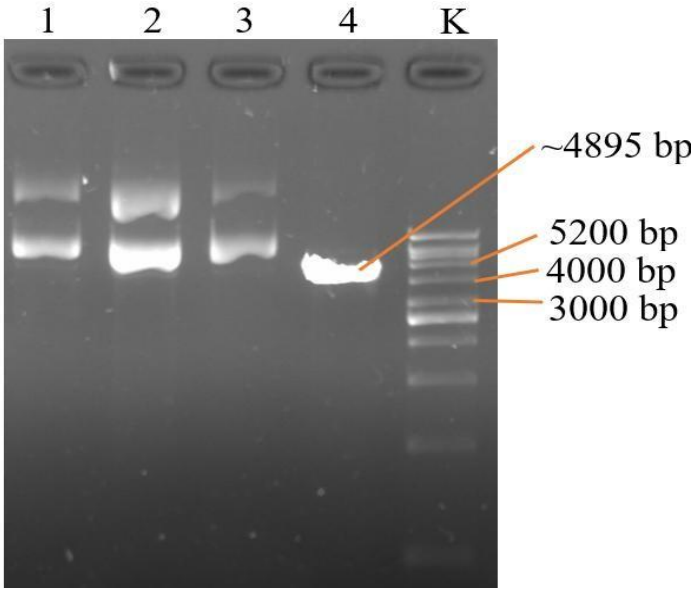


Figure 4.11: Gel electrophoresis of *Eco*RV-digested clone 129. A distinct difference in band position was established between the control, unmodified pASK-KO, and *Eco*RV-digested clone 129. Lane 4 shows that clone 129 was digested with *Eco*RV and the plasmid was linearized to one fragment of 4895 bp.
Lane 1: Undigested pASK-KO
Lane 2: *Eco*RV-digested pASK-KO
Lane 3: Undigested clone 129
Lane 4: *Eco*RV-digested clone 129
Lane K: 1kb DNA ladder

4.11 Sequencing pASK-NrecA-repA-CrecA

The recombinant pASK-NrecA-repA-CrecA was outsourced for DNA sequencing using the R-Ori-Seq primer for insert sequence verification. The sequencing results were aligned using the BLASTN software and compared with the sequence of the expected plasmid pASK-NrecA-repA-CrecA, as shown in appendix B. From the sequencing result, 100% of identities match and no gaps was obtained. See appendix B for the sequence alignment.

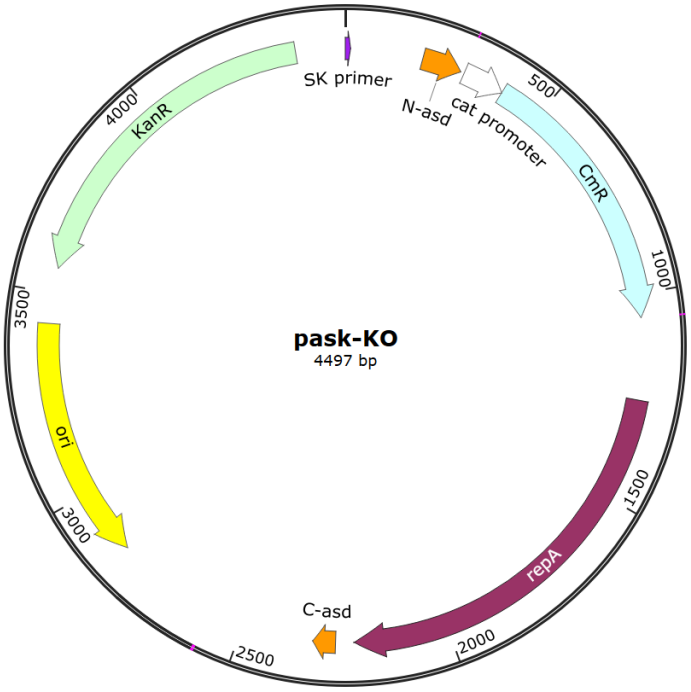
CHAPTER 5

DISCUSSION

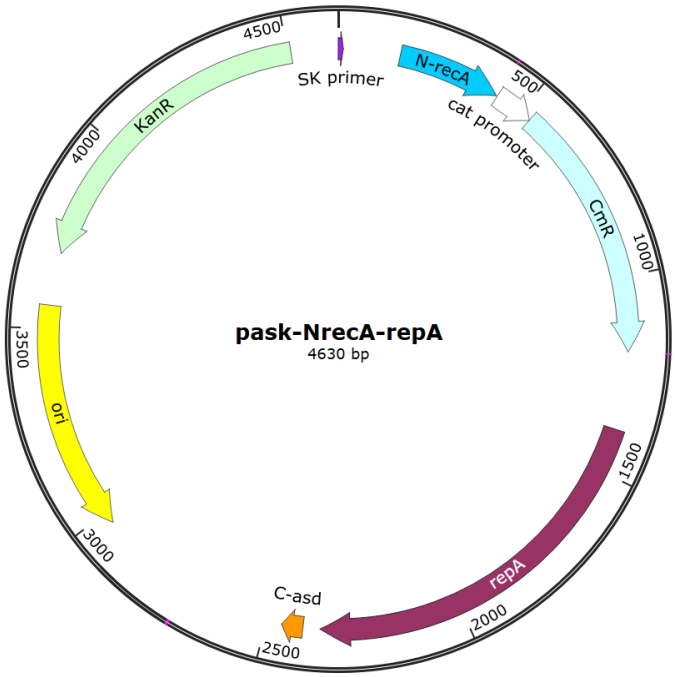
5.1 Cloning amplified N and C terminal of *rec*A into pASK-KO

5.1.1 Cloning of N-*rec*A

N-*rec*A was cloned into the pASK-KO vector, which was verified by vector-specific and orientation-specific PCR. The results of N-*rec*A cloning are shown in Fig. 5.1 (B) whereas the unmodified clone is shown in Fig. 5.1 (A). The digested pASK-KO had an expected band size of 412 bp, and was ligated to the digested vector with a band size of 4218 bp to form a recombinant product of 4630 bp. The experiment yielded a clone containing N-*rec*A in the plasmid and in the correct orientation. This clone was named Clone 99.



(A)

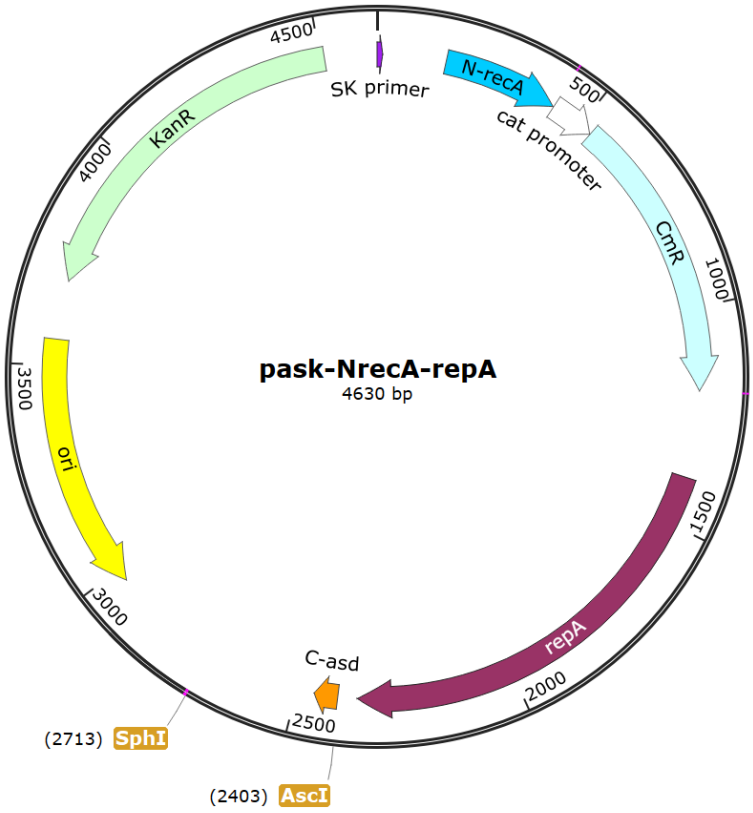


(B)

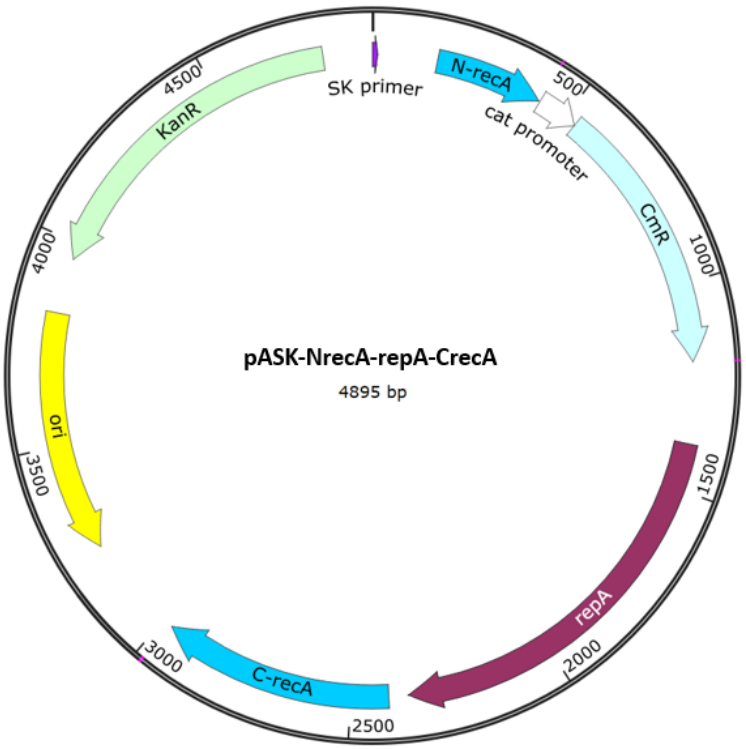
Figure 5.1 Plasmid map of pASK-KO. (A) Original unmodified version of the pASK-KO vector. 5.1 **(B)** shows the modified version of pASK-KO with N-*rec*A cloned into the labelled region.

5.1.2 Cloning of C-*rec*A

Similarly, C-*rec*A was cloned into the sequencing-verified recombinant clone pASK-NrecA-repA. The cloning region of C-*rec*A is indicated in Fig. 5.2 (B) and Fig. 5.2 (A) showed the construct before cloning. This clone was named clone 129.



(A)



(B)

Figure 5.2 Plasmid map of pASK-NrecA-repA. **(A)** illustrates that clone 99 was used as a template plasmid before cloning of C-*rec*A. **(B)** Modified version of pASK-NrecA-repA with C-*rec*A cloned into the labelled region.

5.2 Different types of primers used in colony PCR

The three different types of primers used for screening were vector-specific, orientation-specific, and gene-specific.

One of the vector-specific primers binds to the vector backbone sequence, and the other binds to the gene of interest. This method was employed in the first round of colony PCR screening. This was because the unmodified version of pASK-KO has a recognition site for binding of reverse primer R-PstI-Nasd, while it was absent in the recombinant pASK-NrecA-repA plasmid, as the cloning of N-*rec*A would have disrupted the binding site for primer R-PstI-Nasd. In addition, a non-modified plasmid was amplified in the indicated region, producing an amplicon size of approximately 452 bp (shown in Appendix D). This relatively small amplicon size also greatly reduces the duration of PCR, which accelerates the efficiency of first-round colony PCR screening. In addition, orientation-specific primers were employed in the second-round screening to confirm the insert orientation. The orientation-specific primers yielded a relatively larger amplicon size than previous primers. Therefore, only a few selected clones that produced no bands on the agarose gel were chosen for the second colony PCR screening. This screening method is time-effective. In addition, it also increases the possibility and accuracy of obtaining desired clones. However, colony PCR screening for the C-*rec*A recombinant plasmid only used gene-specific primers for colony PCR. This was due to time constraints during the experiment. Nevertheless, a target band of approximately 580 bp (Appendix E) was obtained from gene-specific amplification, which was later verified as the intended gene of interest.

5.3 Analysis of sequencing result

The sequencing results for N-*rec*A and C-*rec*A cloning are presented in Appendix A and B, respectively. Referring to Appendix A, sequencing showed that N-*rec*A was successfully cloned into the plasmid at the correct position and orientation with 100% identity and no gaps. Additionally, the sequencing outcome for C-*rec*A showed a similar result, with 100% identity and absence of gaps. Furthermore, it is also noteworthy that there is presence of extra sequence 'aaaaaaa' which is in the Chloramphenicol resistance gene. However, this extra sequence did not affect the function of the gene as it confers resistance to chloramphenicol. Appendix C shows *E. coli* TOP10 clone 99 and 129 conferring chloramphenicol and kanamycin resistance to grow on the media supplemented with both the antibiotics together.

5.4 Transformation efficiency of competent cells.

The average transformation efficiency of competent *E. coli* cells prepared using the calcium chloride heat-shock transformation method was 4.95×10^5 CFU/μg. This transformation efficiency is considered relatively low compared to that obtained in other experiments using the electroporation-based method. The low transformation efficiency may be due to several factors, such as the transformation method employed, plasmid size, and the presence of enhancers.

In a study by Zhao et al. (2024), the non-domesticated strain *Bacillus subtilis* GLB191 had a significantly low natural transformation efficiency of approximately 10 CFU/μg. However, the transformation efficiency increased to about $\sim 1.96 \times 10^6$ CFU/μg after optimization. It was also discovered that concentration of glycine, Tween 80, DL-threonine and the voltage used during pulse electroporation may be causally linked to electroporation transformation efficiencies. Further, plasmid size can have an impact on transformation efficiencies. Studies have shown that transformation efficiency declines with increasing plasmid DNA size as high mass DNA can kill bacterial cells (Szostková & Horáková, 1998). Thus, potential methods besides calcium chloride heat-shock methods can include peptide-mediated transformation. It has been introduced to enhance the efficiency to deliver large DNA, low DNA damage, and degradation within the cells (Islam et al., 2019).

On top of that, research by Wang et al. (2020) suggested that chemical transformations such as the heat-shock method with calcium chloride require the supplementation of chemical enhancers for higher transformation efficiency.

Therefore, by considering all the aforementioned factors, the heat shock transformation in this experiment could be further optimized. Nevertheless, despite the resulting transformation efficiency being lower than ideal, the experiment yielded sufficient colony numbers and served its purpose for downstream screening and cloning.

5.5 Challenges and solution

5.5.1 Challenges encountered

A few challenges were encountered in this study. One of the challenges was the absence of visible bands and the presence of only a faint band after agarose gel electrophoresis in the C-*rec*A colony PCR screening. Despite multiple attempts under standard conditions, this problem has persisted. This may be due to the suboptimal annealing temperature for the PCR. The theoretical annealing temperature used in this study was 63°C. This temperature was determined by calculating the average melting temperature of both primers. Finally, faint bands were visualized on an agarose gel when PCR was repeated at 50°C. However, a specific annealing temperature may require further investigation. In addition, the negative control produced a band in one PCR run. This may be due to DNA contamination in the negative control PCR tube and primer solution tube. However, this problem was resolved when a new primer solution and a negative control tube were replaced.

5.5.2 Solution to the problem

The annealing temperature (Ta) of gene-specific PCR amplification for the screening of C-*rec*A can be optimized using gradient PCR. Based on a previous study by Afifatul et al. (2023), gradient PCR was performed to optimize the Ta for specific primers used to analyze GR gene expression in rice. In their study, they tested several sets of primers and selected sets that met the criteria for a

good primer. The selected primer set was chosen for the optimization of Ta using gradient PCR. In gradient PCR, a range of Ta values was tested in many wells. Eventually, Afifatul et al. (2023) identified Ta of 52.2 °C as the most suitable temperature for amplifying the GR gene in rice by comparing gel electrophoresis results in terms of the thickness and intensity of the bands obtained. The study also stated that the ideal Ta should be 5 °C lower than the melting temperature of the primer and ssDNA template. Moreover, optimization of Ta is necessary, as suboptimal Ta may produce undesirable amplification results. Too high a Ta can hinder primer binding to the DNA template, while too low a Ta may result in non-specific amplification, producing unintended PCR products.

Gradient PCR allows multiple Ta values to be tested in a single PCR run. Notably, all PCR conditions, except for Ta, were kept constant. The PCR mix was aliquoted into different wells and each well was subjected to testing with varying Ta. For example, Johnson et al. (2015) conducted gradient PCR including a range of Ta values at 42 °C, 48 °C, 54 °C, and 60 °C. After PCR, the products were analyzed by agarose gel electrophoresis and sequencing to determine the specificity and quality of amplification.

5.6 Future work

After a successful clone consisting of N-*rec*A and C-*rec*A was constructed, the following stage of the experiment was to complete the knockout of *rec*A in *A. tumefaciens*. This involves the transformation of the recombinant clone into *A. tumefaciens* cells. Subsequently, recombinant transformants were selected using chloramphenicol. Those with successful knock-in of the construct will obtain chloramphenicol resistance genes that confer resistance to antibiotics, allowing the bacterial cells to grow in chloramphenicol medium. At the same time, the *rec*A gene is disrupted and loses its function upon knock-in of the construct. The functionality of the disrupted *rec*A gene could be further tested in downstream experiments. Moreover, PCR can be performed to verify gene deletion. Gene-specific primers can be designed to bind to the upstream and downstream regions of deletions. In contrast to the wild-type, the plasmid with *rec*A removed produced a band with a smaller size.

Moreover, the functionality of *rec*A in *A. tumefaciens* should be tested through phenotypic analysis to confirm the functional loss of *rec*A and its downstream effects on *Agrobacterium*-mediated transformation. In a study conducted by Rodrigues et al. (2020), *A. tumefaciens* with *rec*A deficiency made with CRISPR base editing was tested using a drop-dilution assay containing 0.005% (v/v) methyl methanesulfonate (MMS), which is a DNA-damaging agent. These results indicated that the *recA-deficient* strain was hypersensitive to (MMS) and showed reduced growth compared to the wild type. In addition, Adikesavan et al. (2011) demonstrated that the UV-treated *recA-deficient* strain was unable to induce *Lex*A cleavage and the subsequent SOS response, which are critical steps

in DNA repair. This resulted in impaired growth of the *rec*A mutant strain. Therefore, UV radiation was employed in this experiment to confirm the functional deficiency of *rec*A in *A. tumefaciens*.

Moving on, future tests should focus on measuring the transformation efficiency of *rec*A-deficient *A. tumefaciens* strains and their *rec*A^+ counterparts. Although previous work such as Liu et al. (2016) has shown that *rec*A knockout did not hinder the T-DNA transfer mechanism in *Agrobacterium* itself, it will be worthwhile to test transformation efficiency for both transient and stable transformation for various species of plants. This offers a path towards plasmid stability improvement and possibly reduce bacterial growth when co-cultured within plants. A variety of possible experimental designs could be employed to assess the transformation efficiency of *rec*A deficient strains. For example, using reporter genes such as Green Fluorescent Protein (GFP) to quantify transformation efficiency, by comparing colony forming units (CFU) prior to and after co-culturing, or assessing the T-DNA integration rate with PCR or Southern blot (Ayako et al., 2020).

CHAPTER 6

CONCLUSION

In conclusion, the N- and C-terminal PCR amplified from *A. tumefaciens* were cloned into pASK-KO and resulted in pASK-NrecA-repA-CrecA as the final recombinant construct. Orientation-specific amplification and sequencing result has indicated that the N- and C-terminal fragments were ligated in the appropriate orientation. The recombinant plasmids were confirmed by restriction digestion with *Eco*RV. Furthermore, this confirms that the N- and C-terminal fragments were cloned, namely due to the fact that they have a unique restriction site for *Eco*RV. In addition, the sequencing results showed 100 % identity, whereby the cloning corresponded perfectly to the expected plasmid.

Nevertheless, there were some issues that must be addressed in future studies. The first was to perform a gradient PCR to optimize the annealing temperature for optimum amplification. Second, cross-contamination need to be avoided during PCR master mix preparation. In this experiment, a construct was successfully generated that can be used to knock out *A. tumefaciens recA*. Nevertheless, further testing regarding *rec*A functional deficiency, transformation efficiency, and plasmid stability prior to *rec*A knockout experiments may be necessary. This would guarantee the efficiency of future molecular cloning and transformation using *recA*-deficient *Agrobacterium tumefaciens*.

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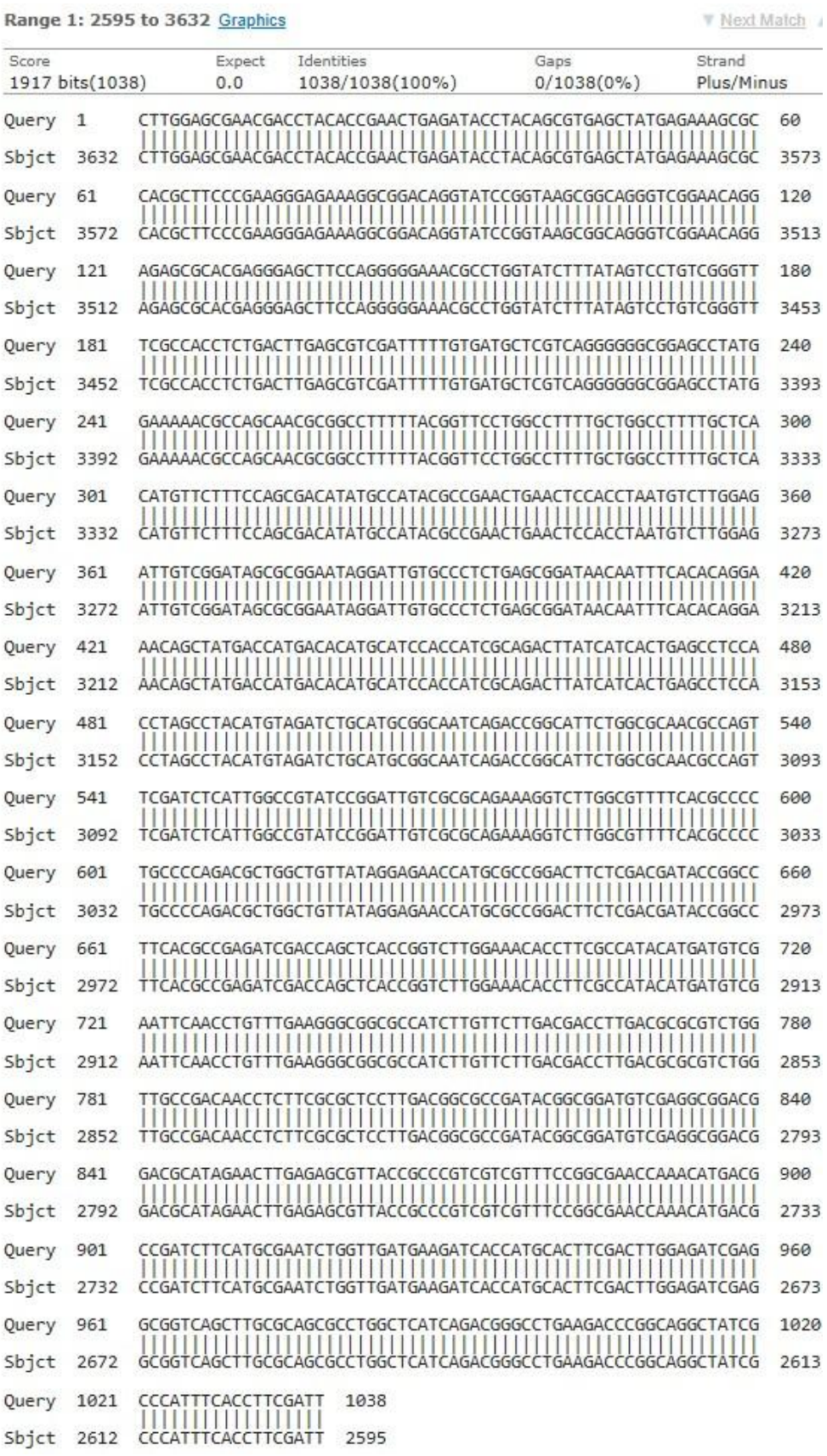
APPENDIX A

Sequence alignment of pASK-NrecA-repA

APPENDIX B



Range 1: 2595 to 3632 Graphics Next Match

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1917 bits(1038)	0.0	1038/1038(100%)	0/1038(0%)	Plus/Minus

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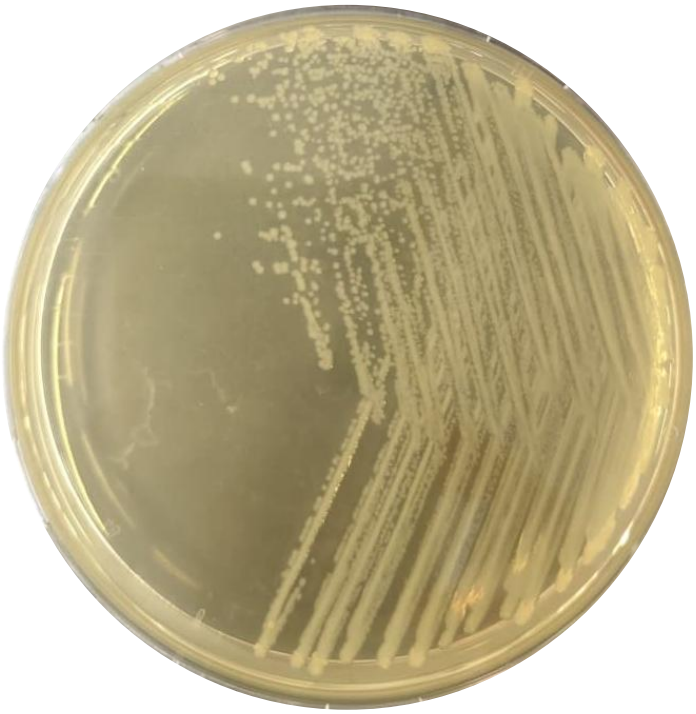
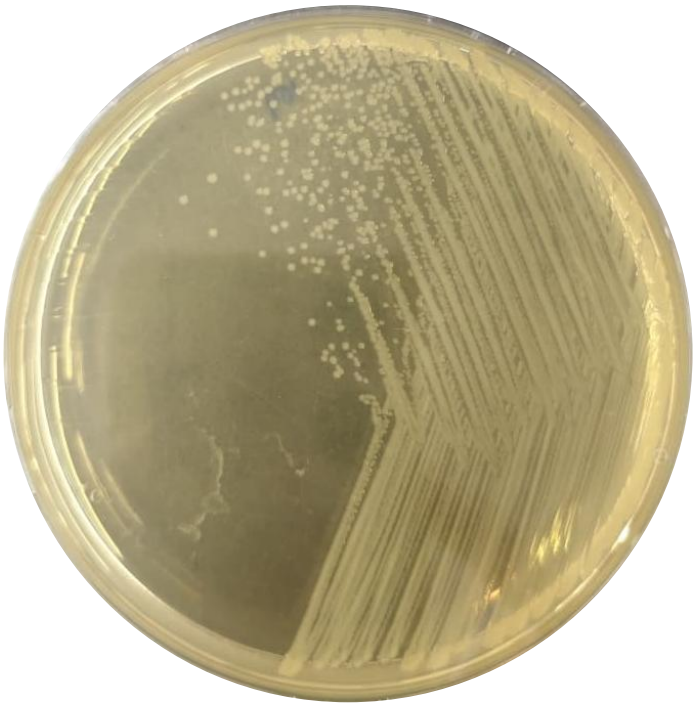
Sequence alignment of pASK-NrecA-repA-CrecA

APPENDIX C



pASK-NrecA-repA (Clone 99) conferred resistance to chloramphenicol and kanamycin



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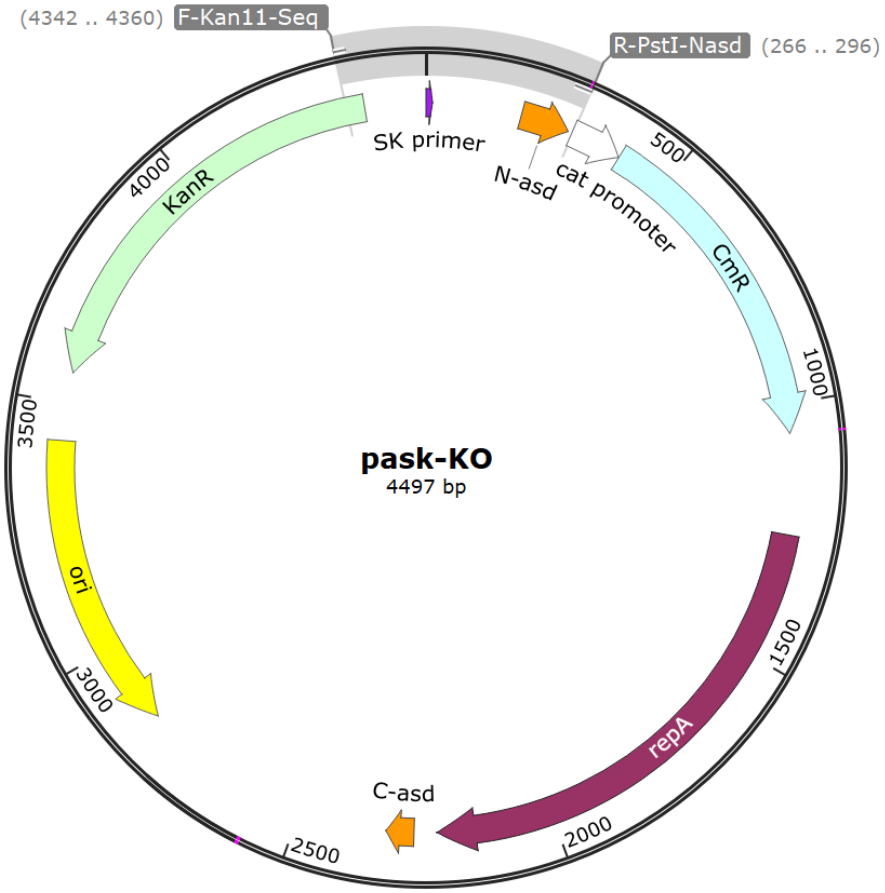
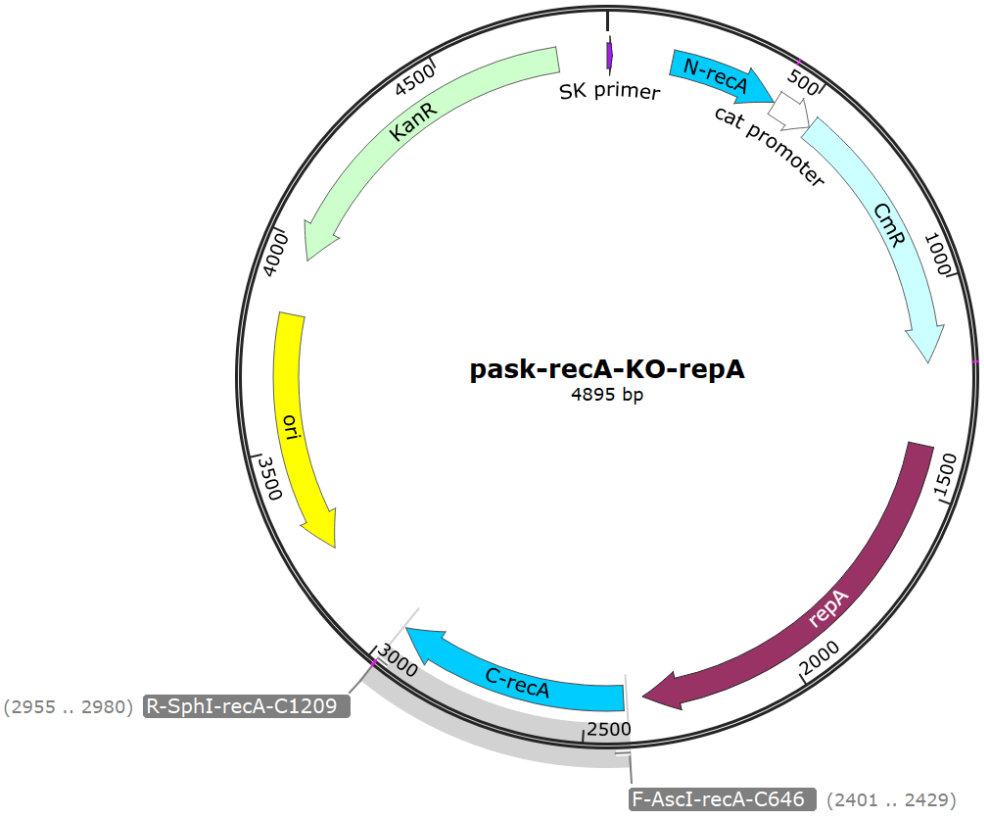
APPENDIX D



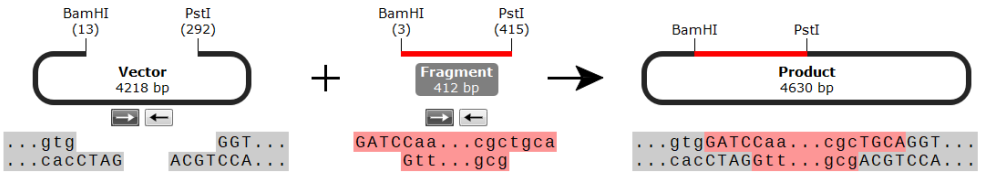
Vector-specific primers used for verifying the pASK-NrecA-repA produce an amplicon of ~452 bp in non-modified clone

APPENDIX E

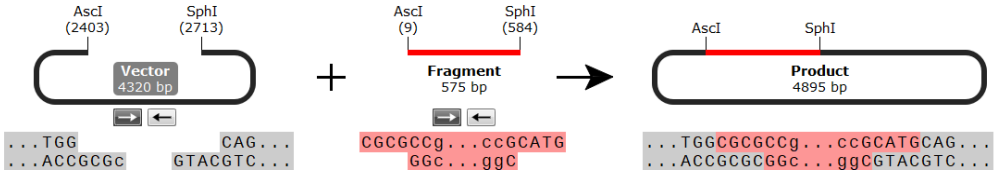


Gene-specific primers used for verifying pASK-recA-repA-CrecA, only desired clone will produce amplicon ~580 bp

APPENDIX F



Restriction digestion reactions for cloning N-*rec*A into pask-KO



Restriction digestion reactions for cloning C-*rec*A into pask-NrecA-repA

APPENDIX G

Table D: The list of chemicals, reagents, antibiotics, and kits used in this final year project with their corresponding manufacturers.

Chemicals	**Manufacturers**
100 bp DNA ladder	Norgen
Agar powder	Titan Biotech Ltd.
Agarose	1st BASE Pte Ltd
*Asc*I restriction enzyme and buffer	Roche
*Bam*HI restriction enzyme and buffer	Roche
Calcium chloride	Bendosen
Ethylenediaminetetraacetic (EDTA)	SIME Scientific
Genomic DNA Extraction Kit	RBC Bioscience®
Glycerol	Systerm Chemicals
Homemade *Taq* polymerase	(unpublished)
Kanamycin antibiotic	BioBasic
PCR Clean-Up and Gel Extraction kit	GeneDireX®

PCR product purification kit.	Macherey Nagel NucleoSpin ® gel kit.
Plasmid extraction kit	NucleoSpin ®
PrimeWay Plasmid DNA extraction kit	1st BASE Pte Ltd
*Pst*I restriction enzyme and buffer	Takara Bio Inc.
Sodium chloride	Chem Soln
SphI restriction enzyme and buffer	Roche
Streptomycin antibiotic	BioBasic
T4 DNA ligase and buffer	Vazyme
Tris base	Fisher Scientific
Tryptone	Pronadisa
VC 1 kb ladder	Vivantis
Yeast extract	Pronadisa

APPENDIX H

Table E: The list of equipment and their corresponding manufacturers used in this final year project.

Equipment	Manufacturer
-20°C Freezer	Liebherr Medline
4°C refrigerator	REMI Scientific
-80°C Freezer	Eppendorf
Analytical balance	Copens Scientific
Autoclave machine	Hirayama
Benchtop mini centrifuge	Sartorius
Fume hood	myLAB^{TM}
Gel imager	Bio-Rad
Incubator	Memmert
Laminar air flow	ESCO
Micropipette	Eppendorf
Microwave	Samsung
Nanodrop spectrophotometer	Thermo Scientific

Oven	Samsung
PCR thermal cycler	Biorad
Power supply	Major Science
Quickspin mini centrifuge	Straits Scientific
Refrigerated centrifuge	Hettich Mikro 22R
Shaking incubator	N-Biotek
UV-light transilluminator	Major Science
Vortex machine	Biocote Ltd
Water bath	Memmert

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It is hereby certified that Victoria Law (ID No: 2102237) has completed this final year project report entitled "MOLECULAR CLONING OF A CONSTRUCT FOR TARGETED RECA GENE DISRUPTION VIA HOMOLOGOUS RECOMBINATION IN *Agrobacterium tumefaciens*" under the supervision of Prof. Wong Hann Ling from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project dissertation in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

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ID Number(s)	2102237
Programme / Course	BACHELOR OF SCIENCE (HONOURS) BIOTECHNOLOGY
Title of Final Year Project	Molecular cloning of a construct for targeted *rec*A gene disruption via homologous recombination in *Agrobacterium tumefaciens*

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Note Supervisor/Candidate(s) is/are required to provide softcopy of full set of the originality report to Faculty/Institut

Signature of Supervisor

Name: Wong Hann Ling

Date: 02.09.2025

Signature of Co-Supervisor

Name: _____

Date: _____

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