T.R.
AKDENIZ UNIVERSITY

USE OF CRISPR/CAS9 SYSTEM TO PRODUCE A MARKER-FREE RICE

Kübra KONTBAY
INSTITUTE OF NATURAL SCIENCES
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

MASTER THESIS

SEPTEMBER 2018
ANTALYA

T.C.

AKDENİZ ÜNİVERSİTESİ

CRISPR/CAS9 SİSTEMİNİN MARKÖRSÜZ ÇELTİK ELDE EDİLMESİNDE KULLANIMININ GÖSTERİLMESİ

Kübra KONTBAY

FEN BİLİMLERİ ENSTİTÜSÜ

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This thesis on 20/09/2018 unanimously accepted by the jury.

Asst. Prof. Dr. Mehmet Aydın AKBUDAK (Supervisor)

Asst. Prof. Dr. Ersin AKINCI

Assoc. Prof. Dr. Ufuk ÇELİKKOL AKÇAY

ÖZET

CRISPR/CAS9 SİSTEMİNİN MARKÖRSÜZ ÇELTİK ELDE EDİLMESİNDE KULLANIMININ GÖSTERİLMESİ

Kübra KONTBAY

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Ana Bilim Dalı

Danışman: Dr. Öğr. Üyesi. Mehmet Aydın AKBUDAK

Eylül 2018; 45

Artan dünya nüfusu nedeniyle yeni ve yüksek verimli tarım ürünlerine ihtiyaç duyulmaktadır. Moleküler ıslah teknikleri ıslah sürecini hızlandırmıştır. Bununla birlikte bitkilerde transformasyon etkinliği çok düşüktür ve bu nedenle bilim insanları transforme olmuş hücre veya dokuları olmanyanlardan ayırmak için markör genler kullanırlar. Markör genler antibiyotik veya herbisitlere direnç sağlayan proteinleri kodlarlar. Bu markör genler genellikle bakteriyel kaynaklardan elde edildiği için markör genler hakında birçok biyogüvenlik endişeleri mevcuttur. Bu endişeler sebebiyle birçok biliminsanı transgenik bitkinin genomundan markör geni uzaklaştırmak için türlü yaklaşımlar denemişlerdir. Fakat bu yaklaşımların çoğu zaman alıcıdır ve bir takım zorlukları vardır. Bu durum markör gen uzaklaştımada yeni ve basit yaklaşımların ortaya çıkmasını gerektirmiştir. Bu çalışmada bölge-spesifik nükleazların son üyesi olan CRISPR/Cas9 tekniğini kullanarak NPT (Neomisin fosfotransferaz) markör genini transgenik çeltik genomundan uzaklaştırılabilirliğini göstermek üzere çalışmalar yapılmış olup transformasyon etkinliğin çok düşük olması sebebiyle pozitif sonuç alınamamıştır.

ANAHTAR KELİMELER: CRISPR/Cas9, Markör gen uzaklaştırma, Bölge-spesifik nükleazlar, Çeltik

JÜRİ: Dr. Öğr. Üyesi Mehmet Aydın AKBUDAK

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ABSTRACT

USE OF CRISPR/CAS9 SYSTEM TO PRODUCE A MARKER-FREE RICE

Kübra KONTBAY

MSc Thesis, Department of Agricultural Biotechnology

Supervisor: Asst Prof. Dr. Mehmet Aydın AKBUDAK

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There is a great demand for new high yield crop varieties due to increased world population. Use of molecular breeding tools has made the breeding process faster. However, transformation efficiency is very low in plants and therefore scientists use marker genes to differentiate the transformed cells or tissue. Marker genes code for a protein which confers resistance to antibiotics or herbicides. Since marker genes generally originate from bacterial sources there are many biosafety concerns about them. Due to these concerns, many scientists have been used numerous approaches to remove marker gene from transgenic plant genome. However, these approaches were time-consuming and had some drawbacks. Thus, there is a need for more simple methods. Here we attempted to remove NPT (Neomycin phosphotransferase) marker gene from transgenic rice genome by using CRISPR/Cas9 technology which is the newest member of site-specific nucleases. However, expected results have not been obtained due to the low transformation efficiency.

KEYWORDS: CRISPR/Cas9, Marker gene excision, Site-specific nucleases, Rice

COMMITTEE: Asst. Prof. Dr. Mehmet Aydın AKBUDAK

Asst. Prof. Dr. Ersin AKINCI

Assoc. Prof. Dr. Ufuk ÇELİKKOL AKÇAY

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Second, I would like to thank my lab mates for their all kind of support. It was easy to keep my motivation high with you guys. Also I am thankful to Dr. Ersin Akıncı and Dr. Ufuk Çelikkol Akçay for their precious advices.

Last but not least I want to endless thanks my family.

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AKADEMİK BEYAN

Yüksek Lisans Tezi olarak sunduğum "Use of CRISPR/Cas9 System to Produce a Marker-free Rıce" adlı bu çalışmanın, akademik kurallar ve etik değerlere uygun olarak yazıldığını belirtir, bu tez çalışmasında bana ait olmayan tüm bilgilerin kaynağını gösterdiğimi beyan ederim.

20/09/2018

Kübra KONTBAY

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

µg	Microgram
µL	Microliter
∞	Infinity Symbol
bp	Base pair
g	Gram
h	Hour
Mg	Miligram
min	Minute
°C	Centigrade Degrees

Abbreviations

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
CIP	Calf-intestinal alkaline phosphatase
PNK	Polynucleotide Kinase
ZFNs	Zinc Finger Nucleases
TALENs	Transcription Activator-Like Effector Nucleases
GUS	β-glucuronidase
NPT	Neomycin phosphotransferase
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeat /CRISPR-associated 9 Endonuclease
DSBs	Double Strand Breaks
sgRNA	Single guide RNA
X-GlcA	5-bromo-4-chloro-3-indolyl β-D-glucuronide

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1. INTRODUCTION

For the first time in 1973, researchers accomplish the integration of a specific gene from a bacterium to another bacterium using restriction enzymes (Cohen et al. 1973) Since this milestone achivement gene cloning techniques have been improved considerably.

Due to exponentially growing world population, currently, there is a big demand for crop varieties providing high-quality, high-yield, feed and fiber. Hence, molecular plant breeding application has been gaining importance every day. In order to use fewer pesticides, fertilizer and water new crop varieties have been continued to be developed. For this aim single or multiple genes need to be integrated into plants or to be excised from plants to gain a certain trait. However, transformation efficiency in plants is very low. Therefore, genome modification applications require selectable marker genes to distinguish transformed cells from the untransformed ones. Marker genes express proteins which confer resistance to a specific substance (substrate) mostly antibiotics or herbicides. For example, HPT gene confers resistance to hygromycin (an antibiotic), NPT gene to kanamycin (an antibiotic) and BAR gene to phosphinothricin and bialaphos (herbicides).

After obtaining transgenic plants the marker gene has no longer function and it is desirable to remove them from transgenic plants.

There are many concerns about cultivation and consumption of crops which contain marker gene. Also, there are some pollution risks which may originate from transgenic plants

On the other hand, marker genes are usually associated with the gene of interest (modified); therefore, they can not be removed by segregation. Moreover, segregation process is not applicable to vegetatively reproduced plants. Thus, removing the marker gene using DNA fragmentation technologies would be a more suitable approach.

Many different molecular methods have been used for removal of marker gene from transgenic plant so far. For example, site- specific recombinases (such as Cre–lox, FLP–*FRT*) have been used successfully for excision of the marker genes (Sreekala et al. 2005; Akbudak et al. 2011).

Also, site-specific nuclease systems are suitable for excision of marker gene. Such as ZFNs, TALENs and most recently CRISPR/Cas9 systems (Townsend et al. 2009; Zhang et al. 2013; Jinek et al. 2012). All these systems induce double-strand breaks at predetermined loci in the genome. Thus, marker gene in the transformation construct can be targeted and removed from the transgenic plant easily.

When comparing these technologies, CRISPR/Cas9 system is the most recently developed sequence specific nuclease. It consists of a single guide RNA (sgRNA) molecule and the associated endonuclease Cas9 to create double-strand breaks (DSBs) at a target site. DSBs are mainly repaired either by nonhomologous end-joining (NHEJ) or by homologous recombination (HR). The repair of DSBs by nonhomologous end-joining (NHEJ) causes deletions and insertions (indels) and these indels eventually may result in gene knock-out due to frame-shift. On the other hand, Homologous recombination (HR) requires sequence homology to repair a DSB. Among these repair pathways, NHEJ is the most common DSBs repair mechanism in many organisms including plants (Bortesi and Fischer 2015). A prerequisite for the CRISPR/Cas9 system is that the target sequence is flanked at the 3' site by a protospacer adjacent motif (PAM) of NGG (Jinek et al. 2012). Several studies have shown that the CRISPR/Cas9 system is much more efficient compared to ZFNs and TALENs (Upadhyay et al. 2013). Furthermore, because the target site is determined by an RNA instead of a protein, the CRISPR/Cas9 system allows for the easier design of new DNA targets, and even multiplexing by using multiple sgRNAs to induce DSBs at multiple target sites at the same time (Belhaj et al. 2015).

In this thesis, CRISPR/Cas9 system was used to remove an antibiotic resistance gene (NPT) from transgenic rice genome. Thus the activity of the CRISPR system has been evaluated for excision of marker gene for the first time.

2. LITERATURE REVIEW

In the plant transformation process, marker genes play a critical role in the identification of transformed cells and tissues. Commonly used marker genes confer resistance to antibiotics or herbicides that suppress the growth of non-transformed cells and allow transformed cells to grow. For this reason, in most transformation processes a marker gene is used with the gene of interest (present in the same construct) (Dan 2011). Otherwise, large-scale screening based on the polymerase chain reaction (PCR) should be performed to isolate transformed plants, but this approach would be inefficient, and expensive (Akbudak et al. 2011).

The transformation efficiency in plants is also very low, making marker gene usage unavoidable. However, there is a general concern about the existence and safety of these marker genes in genetically modified plants. These concerns can be summarized as follows:

Health concerns: The selectable marker gene conferring antibiotic resistance comes from bacterial sources (Yau and Stewart, 2013). If resistance back into bacteria from transgenic plant by horizontal gene transfer; new resistant bacteria may appear in human or animal intestinal organs and these bacteria can no longer be treated with this antibiotics ultimately leading health threats to human and animals.

Environmental concerns: The possibility of gene flow from the transformed plants to wild populations (also known as genetic pollution) is an increasingly considered agricultural and environmental concerns (Akbudak 2010).

Limitation of reuse: The repertoire of antibiotic and herbicide resistance genes used in plant transformation is limited, so the presence of marker gene in the transgenic plant genome limits the reuse of the same marker gene if re-transformation is necessary (Halpin 2005).

Metabolic burden: The presence of the marker gene in the plant genome can sometimes cause metabolic burden in the host plant (Yau and Stewart 2013). For this reason, it is desirable to remove selectable marker genes to ensure wide acceptance of

the genetically modified food products and to eliminate the concerns and risks involved (Woo et al. 2015).

When all above mentioned concerns are considered developing new strategies to remove marker gene from transgenic plants has therefore been an important scientific goal.

Numerous strategies have been developed and reported for excision of a marker gene. Among these there are three basic approaches commonly practiced while producing a transgenic plant with no selectable marker gene.

(1) Avoidance of marker gene. It is possible to not use marker gene but then many PCR has to be conducted to find transformed cells or tissue. This is very laborious and expensive.

(2) Integrating the marker gene and gene of interest into different genetic locus then, and removing the marker gene by segregation (Darbani et al., 2007). Unfortunately, segregation is not an option for vegetatively reproducing plants limiting this strategy for many plants.

(3) Use of molecular approaches (such as the site-specific recombinases and site-specific nucleases)

Cre-Lox and FLP-FRT site-specific recombinases systems have been used successfully to produce marker-free plant previously (Akbudak and Srivastava 2011) In addition, these two systems can be combined and used in the later stages with chemical or heat inducible promoters (Srivastava and Ow 2004).

Site-specific nucleases such as ZFNs and TALENs can be used for excision of marker gene from transgenic plant genome.

The most recently developed site-specific nuclease is the CRISPR/Cas9 system (Figure 2.1). This system has accelerated plant genome engineering since its emergence. CRISPR/Cas9 system consists of a single guide RNA (sgRNA) molecule and an associated endonuclease Cas9 to generate double-strand breaks (DSBs) at the intended genomic loci. A 20bp single guide RNA is complementary to the target site. Cas9

protein and sgRNA form a complex and then create double-strand breaks at the target region. The only prerequisite for recognition of the target region by Cas9/sgRNA complex is the requirement for an NGG sequence that called PAM (Protosapter Adjacent Motif) at the 3 'end of the target region (Gasiunas et al. 2012; Mahfouz et al. 2014).

A rice line used in this master project was a homozygous transgenic line which contain UBI-NPT-GUS (Ubiqitin- Neomycin phosphotransferase- β-glucuronidase) locus in its genome (Figure 2.2). NPT gene is a marker gene and it confers resistance to kanamycin antibiotic to the transgenic rice genome. There are appropriate PAM sequences to the right and left of the NPT gene. A CRISPR transformation vector prepared carefully by cloning the sgRNA which targeting the right and left site of the NPT gene. Once the marker gene, NPT, is removed using CRISPR/Cas9 system, the UBI promoter will be able to drive the GUS gene. As a result, GUS staining would reveal if the marker gene is removed from the target locus.

Although there is an article proposing that CRISPR technology can be used successfully to remove GUS reporter gene from the plant genome (Srivastava et al. 2017), there is no study empirically showing that the antibiotic resistance gene can be removed from transgenic rice by CRISPR system.

2.1 Demonstration of Figures

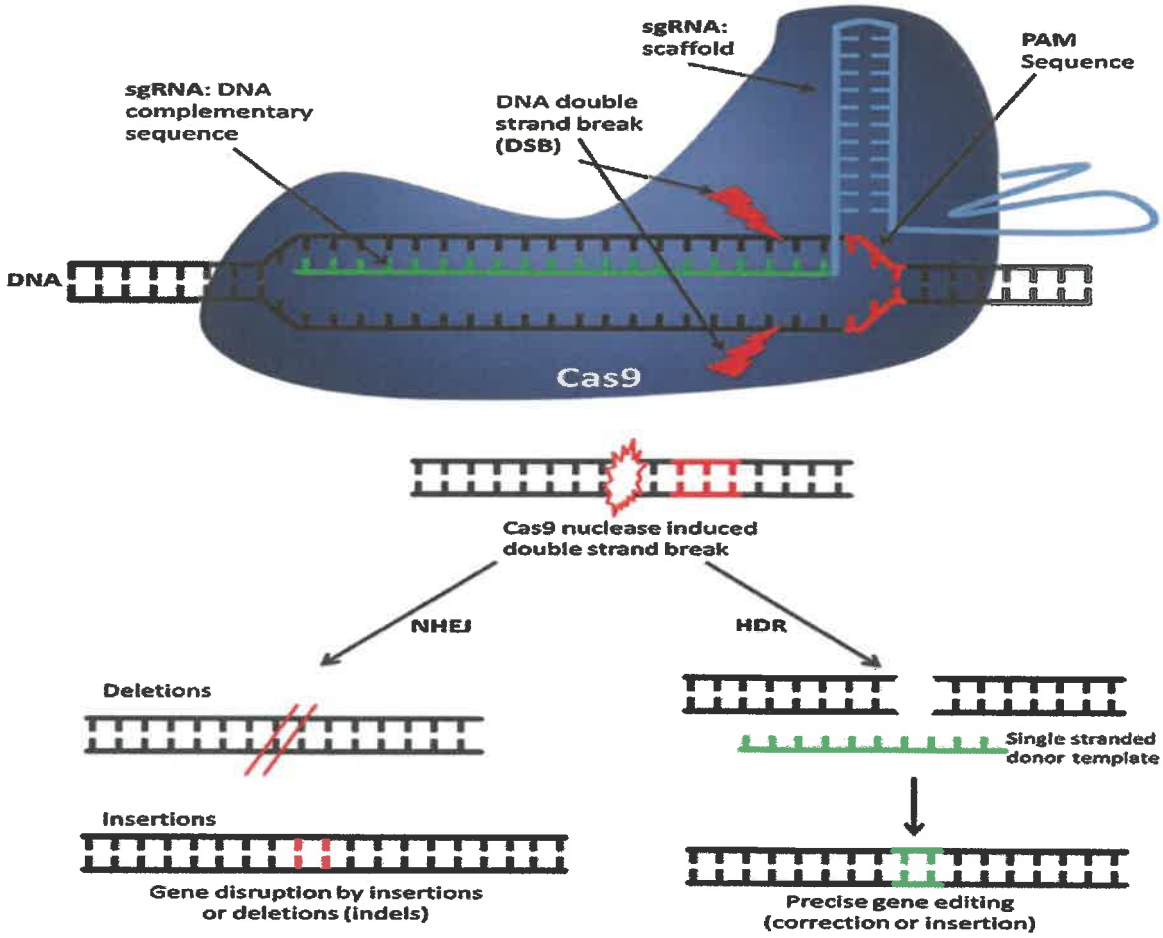

Figure 2.1. CRISPR/Cas9 system (Pellagatti et al. 2015)

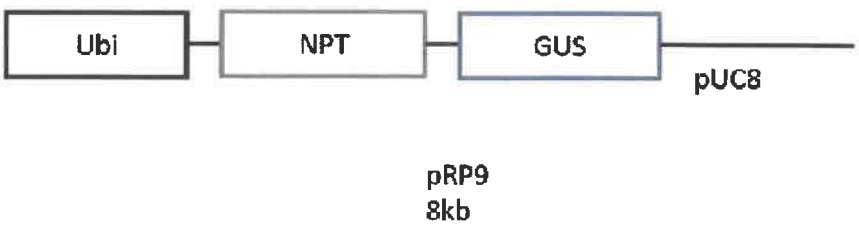

Figure 2.2. Transgenic loci in rice genome.

3. MATERIALS AND METHODS

3.1 Plant Material

In this study, we used a transgenic *Oryza sativa* (Nipponbare) line carrying pRP9 locus as a plant material. To verify the transgenic loci we set PCR with primers shown in Table 3.1

Table 3.1. Primers used for transgenic loci verification PCR

Primer name	Sequence of forward	Sequence of reverse
UBI KANR	5'-TCTACTTCTGTTCATGTTTGTG -3'	5'-CTCGATGCGATGTTTCGCTT -3'
KANF GUSR	5'- GCATCGCCTTCTATCGCCTT -3'	5'-AATTACGAATATCTCGATCGG -3'

3.2 CRISPR/Cas9 Transformation Vector Construction

To construct a CRISPR tranformation vector we followed two different protocols (Xie et al. 2014 and Xie et al. 2015).

3.2.1 Preparation of first CRISPR transformation vector (pRGEB31)

pRGEB31 was a gift from Yinong Yang (Addgene plasmid # 51295). Plasmid map of pRGEB31 was shown in Figure 3.1. Only one sgRNA has been integrated into this vector.

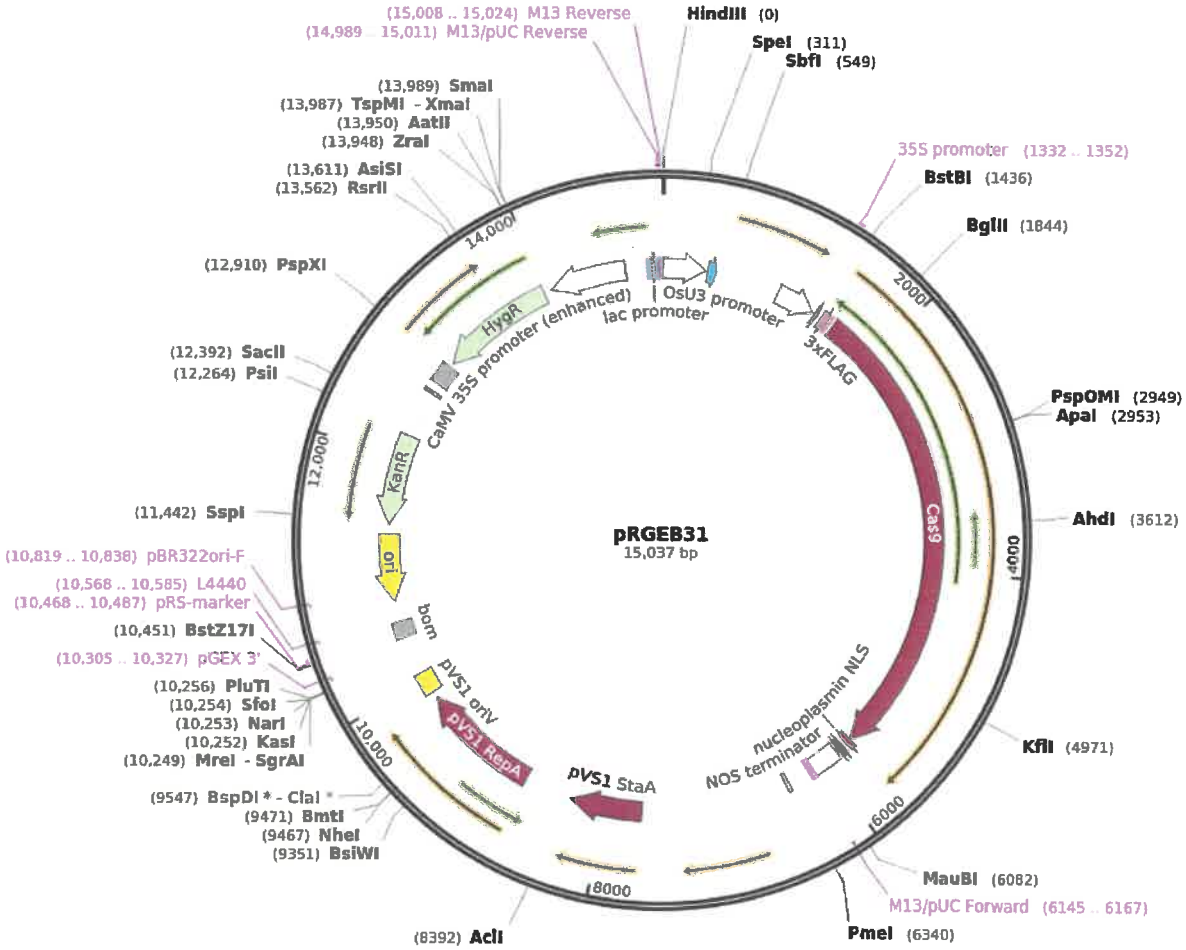

Figure 3.1. Plasmid map of pRGEB31 vector

3.2.1. Target site selection

There are FRT sites on the left and right of the NPT sequence. These sequences contain PAM (AGG) sequence which is prerequisite for CRISPR/Cas9 system. For the second transformation vector (pRGE32) we designed two different sgRNA. These two sgRNAs target left and right flanking sequences.

Target sequence: GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

3.2.2. Design of sgRNA for the pRGEB31 CRISPR transformation vector

For the first transformation vector, sgRNA was designed according to protocol of Xie et al. 2014 (Table 3.2). Briefly, sgRNA synthesied as a two seperate oligos which contain adaptor sequences for cloning. Adaptor sequences are: 5'-GGCA-3' and 5'-AAAC-3'.

Forward FRT: 5'-GGCA-T-C-C-T-A-T-T-C-T-C-T-A-G-A-A-A-G-T-A-T-3'

Reverse FRT: 5'-AAAC-A-T-A-C-T-T-T-C-T-A-G-A-G-A-A-T-A-G-G-A-3'

3.2.3. Construction of the gRNA-Cas9 plasmid

1) pRGEB31 vector was digested by BsaI enzyme (New England Biolabs)

Table 3.2. Components of the digestion reaction

pRGEB31	2µg
10x NEB Buffer 4	2µl
10x BSA	2µl
Bsa I (NEB)	1µl
Add H_2O to	20µl
Incubated at 37 °C for 3 h.	

2) 0.5 µl of CIP were added to dephosphorylate the pRGEB31 and incubated at 37 °C for 30 min.

3) Digested vector were purified using the Monarch PCR & DNA Cleanup Kit (NEB)

3.2.4 Preparation of DNA oligo-duplex

Table 3.3. Components of the oligo-duplex reaction

Forward FRT	1µl
Reverse FRT	1µl
10x T4 DNA ligase Buffer	1µl
T4 PNK (NEB)	0.5µl
H_2O	6.5µl

4) Following thermal cycler program used

Table 3.4. Thermal cycler program

37 °C	60min
95 °C	10 min

5) Before ligations oligo-dublex were diluted (1:200)

6) Oligo-dublex were ligated into digested vector.

7) Reaction mixture was incubated at room tempature (25°C) for 4 hours

Table 3.5. Component of the vector construction reaction

BsaI digested vector	~50ng
Oligo-dublex	1µl
10x T4 DNA ligase Buffer	0.5µl
T4 ligase (NEB)	1µl
H_2O to	5µl

8) E. *coli* DH5α competent cells were transformed using 1 µl of ligation product.

9) 3 colonies (Figure 3.2.) were inoculated in LB medium with 50 µg/ml kanamycin.

10) Plasmids were purified from the transformed DH5α cells using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific)

11) pRGEB31- FRTgRNA plasmid constructs were confirmed by Sanger sequencing using M13R (-48) primer.

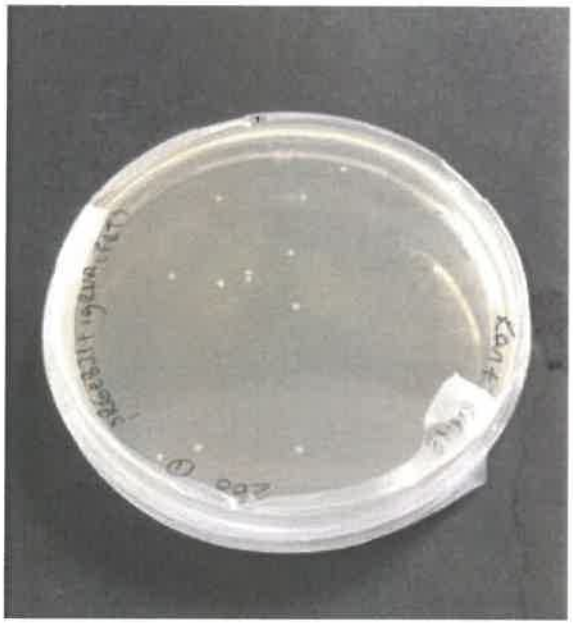

Figure 3.2. The colonies after transformation.

3.2.1 Preparation of second CRISPR transformation vector (pRGE32)

pRGE32 was a gift from Yinong Yang (Addgene plasmid #63159). Plasmid map of pRGE32 was shown in Figure 3.1. Two different sgRNAs have been integrated into this vector.

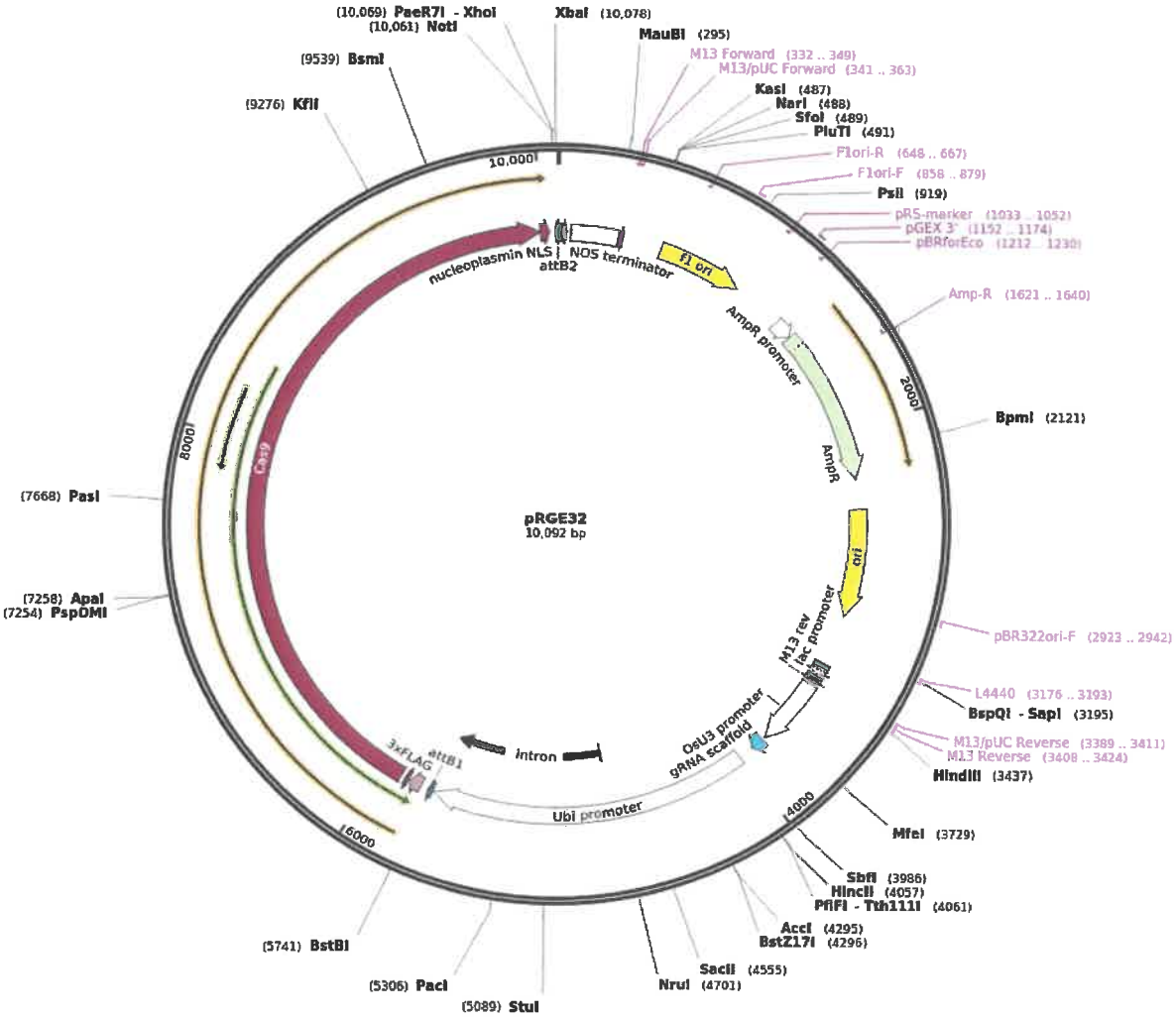

Figure 3.3. Plasmid map of pRGE32 vector

Design of sgRNA for the pRGE32 CRISPR transformation vector

For the second transformation vector, sgRNA was designed according to protocol of Xie et al. 2015. This cloning approach benefit from polycistronic tRNA-gRNA (PTG) genes. Golden Gate assembly has been used to synthesis of PTG genes. Preparation of CRISPR PTG genes included many steps.

Primers design to amplify gRNA-tRNA parts

Primers were used as shown in Table 3.6. Primers contain 4-bp overhangs as required for Golden Gate (GG) assembly. These overhangs are necessary for ligation of DNA parts after *Bsa*I digestion.

Table 3.6. Primers used for construction of gRNA-tRNA

Primer names	Sequences of primers for GG assembly
gFRT 1-F	5' -TAGGTCTCCTCCTCTAGGATCGTTTTAGAGCTAGAA-3'
gFRT 1-R	5'- CGGGTCTCAAGGATCGTTTCCTGCACCAGCCGGG-3'
gFRT 2-F	5'- TAGGTCTCCGAATTGGATCCCGTTTTAGAGCTAGAA-3'
gFRT 2-R	5'- CGGGTCTCAATTCGAAGTTCCTGCACCAGCCGGG-3'
L5AD5-F	5'- CGGGTCTCAGGCAGGATGGGCAGTCTGGGCAACAAAGC ACCAGTGG-3'
L3AD5-R	: 5'- TAGGTCTCCAAACGGATGAGCGACAGCAAACAAAAAAA AAAGCACCGACTCG-3'

Table 3.7. Component of the PCR for PTG assembly

pGTR plasmid (Addgene #63143)	0.1ng
5X Phusion HF buffer	10 µl
dNTPs	1 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
Phusion enzyme (NEB)	0.5 µl
H_2O	x µl
Total volume	50 µl

Table 3.8. Following thermal cycler program used

Temparature	Time	Cycles
98°C	2 min	1
98°C	10 sec	35
50°C	20 sec	
72°C	20 sec	
72°C	2.5 min	1
4°C	Hold	1

Subsequently, purification of PCR products has been done by QIAquick PCR purification kit. PCR products of two seperate amplification reactions were ligated by GG assembly (Table 3.9). GG reaction program is shown in Table 3.10.

Table 3.9. Ligated PCR products reaction components

Purified PCR products	25-50 ng
2X T7 DNA ligase buffer	10 µl
Bovine Serum Albumin	2 µl
Bsa I enzyme (NEB)	0.5 µl
T7 DNA ligase enzyme (NEB)	0.5 µl
Total volume	20 µl

Table 3.10. GG reaction program

37°C	5 min
20°C	10 min 40 cycles
20°C	1 hour

The product of this reaction was diluted with 180 µl of H_2O and used as a template for amplification by using S3AD5-F and S3AD5-R primers (Table 3.11 and Table 3.12).

Table 3.11. The sequence of primers which used in GG assembly product amplification

Primer name	Sequence of primer
S3AD5-F	5'- CGGGTCTCAGGCAGGATGGGCAGTCTGGGCA-3'
S3AD5-R	5'-TAGGTCTCCAAACGGATGAGCGACAGCAAAC-3'

Table 3.12. Component of PCR reaction

Ligation product	1 µl
DreamTaq DNA Polymerase	12 µl
S3AD5-F	1 µl
S3AD5-R	1 µl
H_2O	10 µl
Total	25 µl

PCR product of this reaction was purified by using QIAquick PCR purification kit. The cleaned PCR product was digested with *Fok* I enzyme at 37°C. Digested PCR product was separated in 1% agarose gel. Expected top band excised from the gel and purified. FokI digested fragment and BsaI digested pRGE32 vector ligated with T4 DNA ligase. Other steps have been done same as first CRISPR transformation vector (pRGEB31).

3.3 Callus Induction

Rice callus produced from seed (Figure 3.3). Rice seeds were sterilized in commercial bleach (4.5% sodium hypochlorite), washed 3 times in distilled water and dried in sterile filter paper. Steril rice seeds were cultured on callus induction medium (Table 3.13) for 6-10 weeks.

Table 3.13. Recipe of callus induction medium

All components were dissolved in 1 liter distilled water. pH was adjusted to 5.8. Callus Induction Medium was used after autoclaved.	N6 basal salt mixture (g)	3.98
	Myo-inositol (g)	0.01
	Casaminoacids (g)	0.3
	L-Proline (g)	5.75
	2,4-D (10mg/ml) (µl)	200
	Sucrose (g)	30
	N6 vitamin (1000x) (1ml)	1
	Agar (g)	7

Figure 3.4. Rice Callus

Table 3.14. Recipe of bombardment medium

All components were dissolved in 1 liter distilled water. Bombardment Medium used after autoclaved.	N6 basal salt mixture (g)	3.98
	Myo-inositol (g)	0.1
	Casaminoacids (g)	1
	Sorbitol (g)	63
	2,4-D (10mg/ml) (µl)	200
	Sucrose (g)	30
	N6 vitamin (1000x) (1ml)	1
	Agar (g)	7

3.4 Bombardment of CRISPR Transformation Vectors

Rice calli or leaves were placed onto bombardment medium (Table 3.14) for 4 h before bombardment (Figure 2.3)

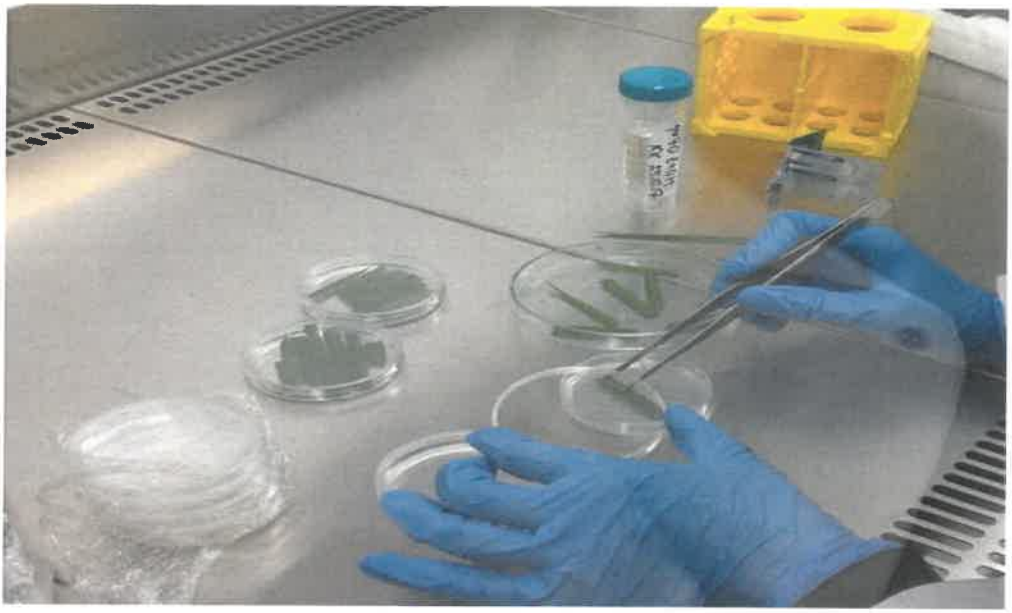

Figure 3.5. Transgenic rice leaves placed onto bombardment medium

3.4.1 Biolistic Bombardment

Bombardment of rice leaves and callus has been done as described in Feng et al. 2017 (Figure 3.5).

Figure 3.6. Preparation for bombardment of rice leaves and callus

3.5 GUS Staining

For GUS staining assay performed according to the β-Glucuronidase Reporter Gene Staining Kit (Sigma-Aldrich) instructions. Briefly, calli or leaf explants were covered with a solution which contains X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and incubated at 37°C for 3-4 days.

3.6 Polymerase Chain Reaction (PCR)

After bombardment with first transformation vector (pRGEB31) some of the calli have been placed on callus induction medium supplemented with Hygromycin (50mg/ml). DNA was extracted from putative transformant calli and PCR was conducted to amplify Cas9 gene. Primers are shown in Table 3.15 and gel photograph is shown in Figure 4.9.

Table 3.15. The sequence of primers which used in Cas9 gene amplification

Primer name	Sequence of primer
Cas9-F	5'-AGTACAAGGTGCCCAGCAAG-3'
Cas9-R	5'-GTCGATCCGTGTCTCGTACA-3'

4. FINDINGS

4.1. Verification of Transgenic Rice Line

Firstly, PCR perfomed to verify if the transgenic rice line carriying target loci (Figure 4.1)

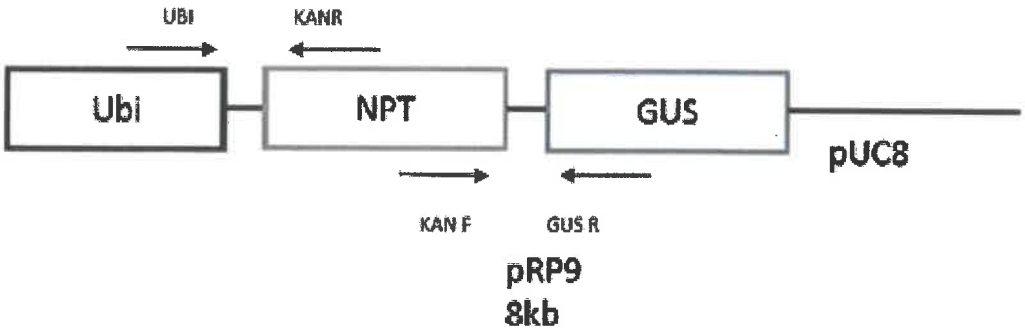

Figure 4.1. Illustration of transgenic locus present in the transgenic rice genome. Right and left sites of the NPT gene have been targeted.

Gel electrophoresis result demostrated that target loci present in the transgenic rice genome (Figure 4.2)

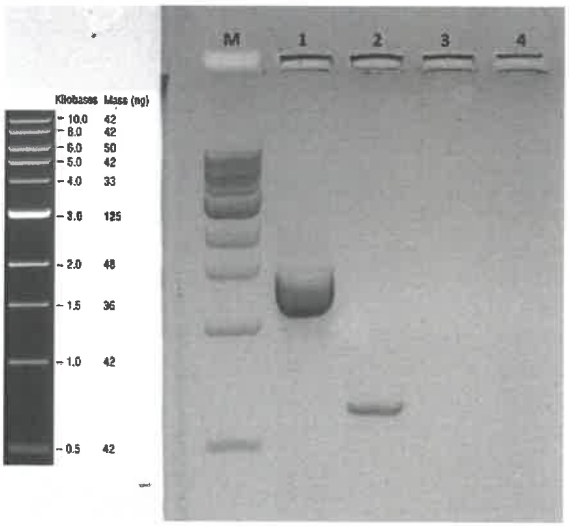

Figure 4.2. Gel electrophoresis of PCR products. Line 1 amplified with UBI and KANR primers. Line 2 shows the PCR product of KANF and GUSR primers. M: 1 kb marker. Line 3 and 4 nontemplate control of line 1 and 2 respectively.

4.2. Confirmation of Transformation Vectors

Presence of sgRNA(s) in the CRISPR transformation vector confirmed by Sanger sequencing (Figure 4.3 and Figure 4.4)

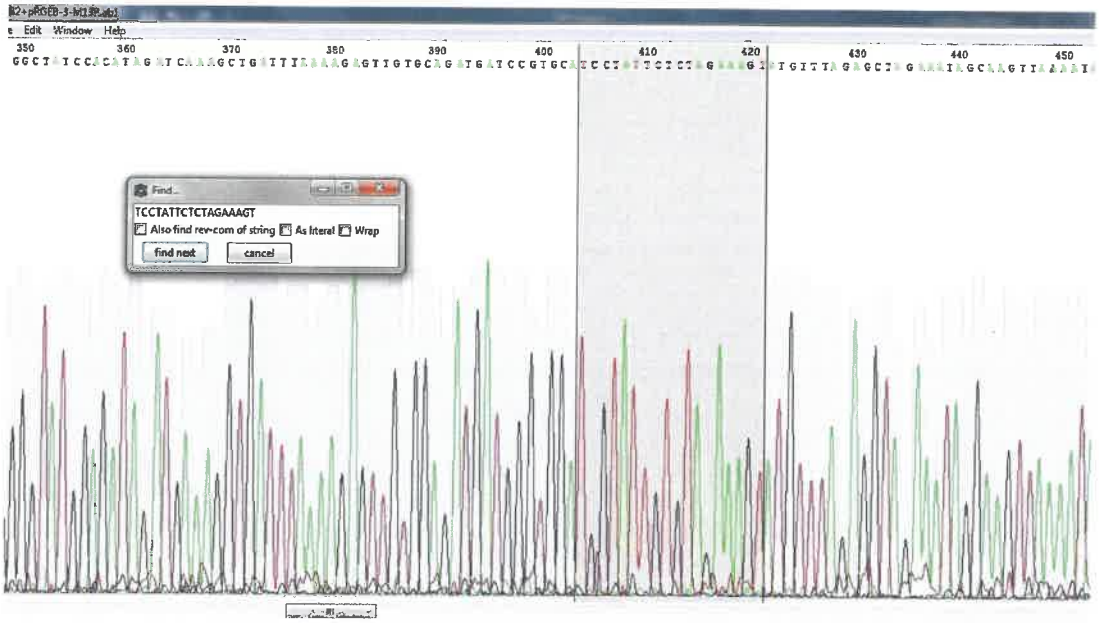

Figure 4.3. Confirmation of the first CRISPR transformation vector (pRGEB31).

GCCGGTGCCAGGCATTACGCCAGCTTAGGATCTTTAACATACGAACAGATCACTTAAAGT
TCTTCTGAAGCAACTTAAAGTTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCA
GTCAGGGACCATAGCACAAGACAGGCGTCTTCTACTGGTGCTACCAGCAAATGCTGGAA
GCCGGGAACACTGGGTACGTTGGAAACCACGTGATGTGAAGAAGTAAGATAAACTGTAG
GAGAAAAGCATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCC
GGCCCATTACGCAATTGGACGACAACAAAGACTAGTATTAGTACCACCTCGGCTATCCAC
ATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGCAACAAAGCACCAGT
GGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGT
GCAGGAAACGATCCTCTAGGATCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCT
AGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTA
GTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGGA
ACTTCGAATTGGATCCCGTTTTAGAGCTAGAGTCGGTGCTTTTTTTTTTTGTTTTAGAGCTA
GAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTC
GGTGCTTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTTAAAATAAAGCTAGTCCGTTTTTA
GCGCGTGCATGCCCTGCAAGTCACAAATTCGGTCAAGCGGAAGCCAGCGCGCCACCCCA
CGTCAGCCAAATACGGAAGCGCGGGGTTGGACGCGTCACCCGGTCCTAACGGCGACCAA
CAATCCAAGTTGAGAATACGAT

FRT sgRNA1: GGAAACGATCCTCTAGGATC

FRT sgRNA2: GGAACTTCGAATTGGATCCC

Figure 4.4. Confirmation of the second CRISPR transformation vector (pRGE32).

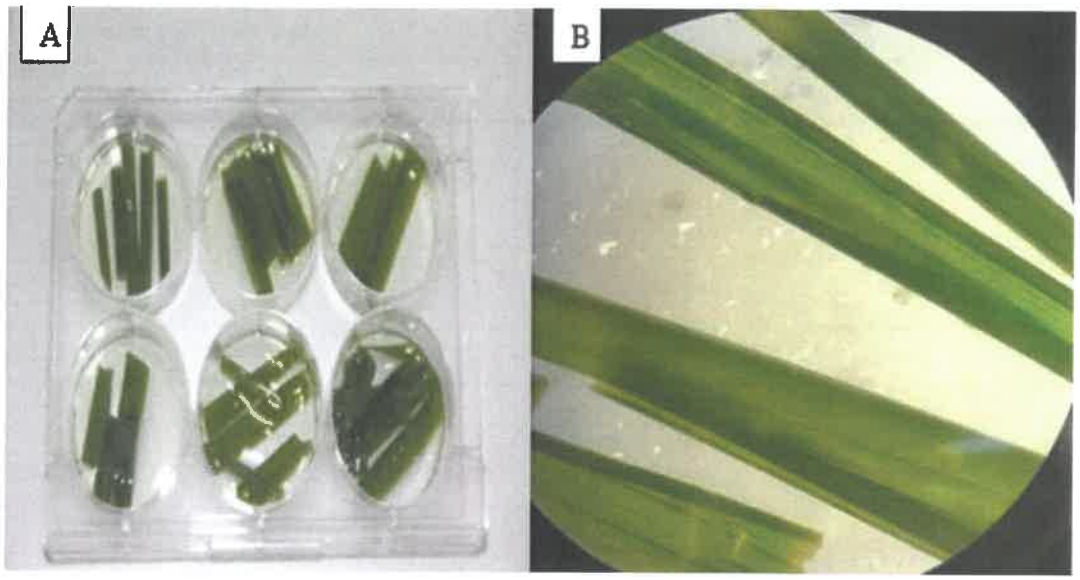

Figure 4.5. A) GUS staining of bombarded leaves of transgenic rice. B) Bombarded leaves after GUS staining under stereo microscope.

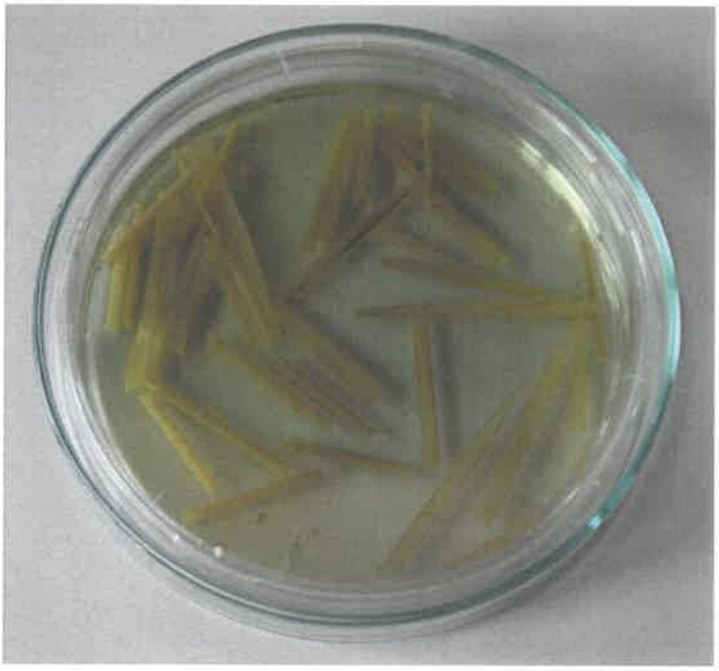

Figure 4.6. Bombarded leaves after removal of chlorophll in ethanol. No blue dot has been detected.

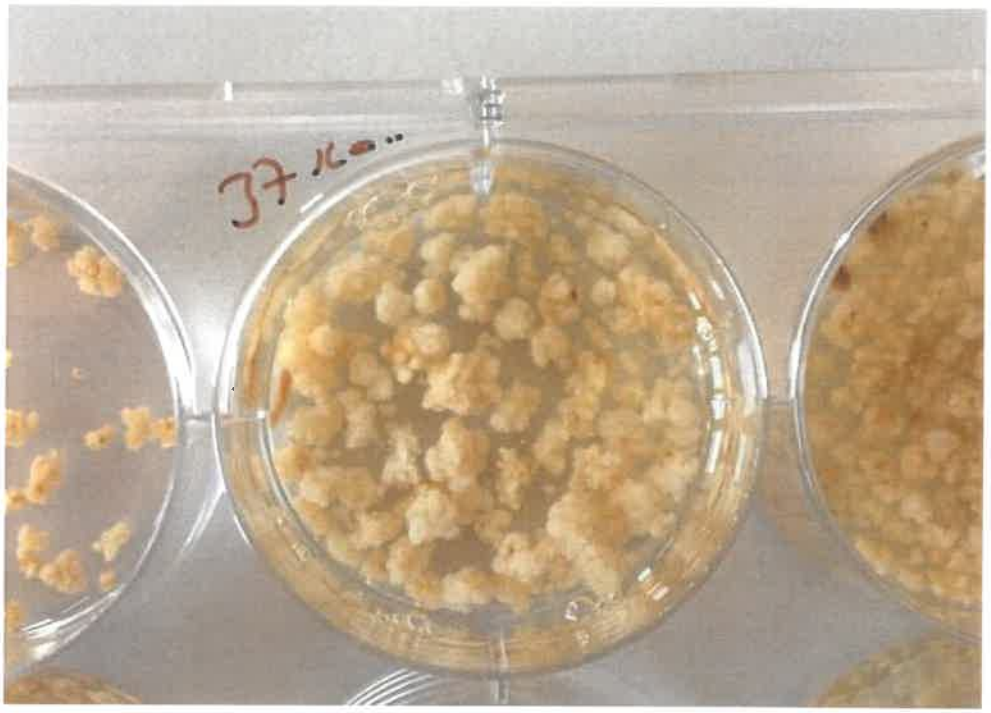

Figure 4.7. Bombarded rice callus after GUS staining. No blue dot has been detected.

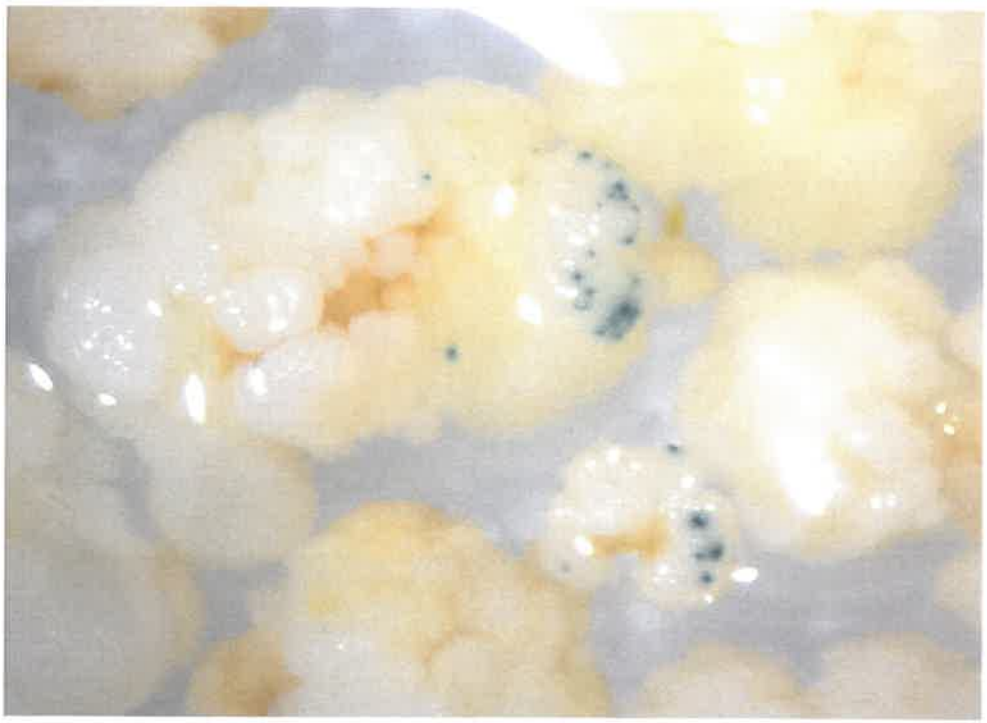

Figure 4.8. Rice callus bombarded with pCAMBIA 1301 as a positive control. Callus photographed after GUS staining.

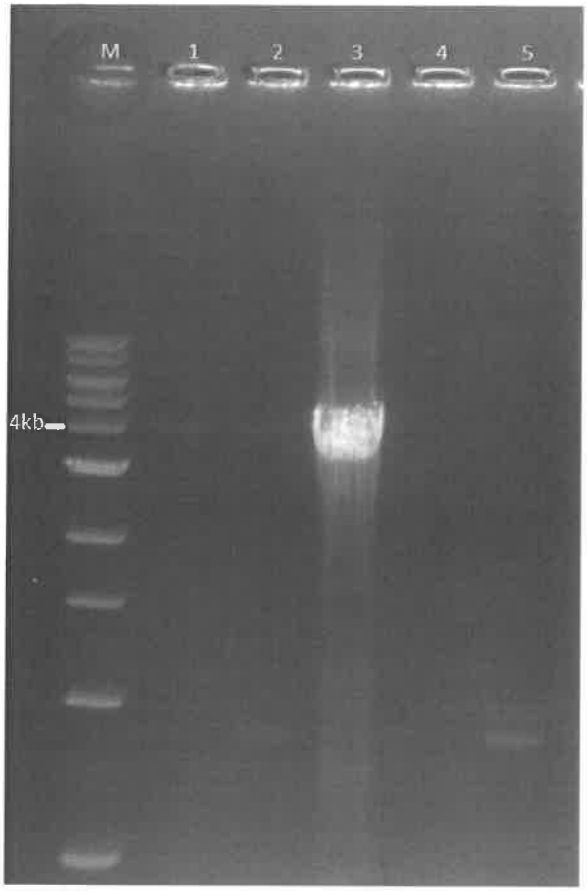

Figure 4.9. PCR analysis for the presence of Cas9 gene. Line 1-2 shows putative transformants. Line 3 is the positive control. Line 4 is the negative control. Line 5 is non-transformed negative control.

5. DISCUSSION

The aim of this study is to remove marker gene from transgenic rice line. Once the transgenic plant has been separated from the non-transgenic individuals there is no need for the marker gene used.

A number of different strategies have been used to remove marker genes. However, most of these strategies were very time-consuming and laborious. Also, some of the strategies had a drawback and need for further improvement. Therefore there is a need for a new and easy strategy.

In this master study, we particularly target the NPT (Neomycin phosphotransferase) marker gene which confers resistance to kanamycin. For this aim CRISPR/Cas9 system was used.

CRISPR/Cas9 technology is the last member of site-specific nucleases and this system has been widely used for several purposes such as gene knock-out and gene insertion.

We designed two different CRISPR transformation vectors by following protocols (Xie et al. 2014; Xie et al.2015). gRNAs in the CRISPR transformation vectors were verified by Sanger sequencing.

The CRISPR transformation vectors which carry sgRNA(s) for NPT targeting were bombarded onto healthy transgenic rice leaves or rice callus. After GUS staining no blue dots were observed. The reason for that would be insufficient transformation efficiency because we were not able to see many blue dots for our positive control either. In addition, some of the calli have been placed on a selective medium to differentiate the transformants. PCR analysis for Cas9 gene amplification resulted in very weak bands. This suggests that most of the putative calli were chimeric.

Moreover, non-specific cuts may possible. Even though, it has been indicated that Cas9 usually generate double-strand break 3-4 bp upstream of the PAM sequence unintended large deletions may occur. On the other hand UBI promoter, NPT gene (target gene) and GUS gene sequence pretty close each other and we tried to make the genetic modification at this really narrow location in the transgenic rice genome. Any

unintended indels cause by CRISPR/Cas9 may result in frame-shift. In this case, even though NPT gene removed, the UBI promoter could not be able to drive the GUS gene. Another possibility is that NPT gene may religated immediately after the excision. Also nuclear localization sequence (NLS) necessary for Cas9 transportation into the nucleus but it is already present at the transformation vectors. Thus Cas9 protein must be transferred into the nucleus. All of these reasons may possible explanation for why we were not able to excise the NPT gene.

Elimination of marker gene from transgenic plant genome would alleviate biosafety concerns about genome edited plants. Researchers used many different approaches to obtain marker-free plants. Some scientist avoided using marker gene. Even though it is laborious and expensive PCR can be conducted after transformation with putative transformants to find transformed plants. Bhatnagar et al., (2010) used PCR to identify transformed peanut shoots. Doshi et al., (2007) obtain transgenic triticale and wheat plants without using marker gene with a frequency of 0.93% and 1.55% respectively. Li et al., (2009) also set PCR to produce marker-free transgenic tobacco plants. De Vetter et al., (2003) used PCR strategy to select transformed potato shoots.

Co-transformation strategy has been used by many scientists (Miki et al. 2004). This approach included co-transformation of marker gene with transformation construct (gene of interest). For example, Cas9-sgRNA construct would be in the same vector or separate ones with marker gene. Afterward marker gene would be removed from genome edited plants genome by segregation. However, segregation process takes a long time and not convenient for vegetatively propagated plants.

Many scientists have been reported that site-specific recombinases can be used for removal of marker genes. For example; FLP-FRT and Cre/*lox* site-specific recombination systems have been successfully used to excise the marker gene (Arumugam et al. 2007; Akbudak et al. 2011) It has been reported by Chakraborti et al., (2008) Cre/*lox* site-specific recombination systems can be used for removal of nptII marker gene and finally produce marker-free insect resistant tobacco plants. Also, Bala et al., (2013) used Cre/*lox* mediated recombination system to produce marker-free (hygromycin resistant gene free) insect resistance mustard plants.

Chemical or heat inducible site specific recombinases have been used effectively (Zuo et al. 2001; Wang et al. 2005; Sreekla et al.,2005; Akbudak et al. 2011)

Among the site-specific nucleases, ZFNs and TALENs can be used for removal of marker gene however there is no study has been reported so far.

Srivastava et al. 2017 have shown that GUS reporter gene can be removed from transgenic rice genome by using CRISPR technology. This group used dual sgRNA to target GUS reporter gene and they have successfully removed GUS gene from the transgenic rice genome. Although we used the same plasmids, we were not able to excise NPT gene from the rice genome.

All in all, our approach to excise the NPT gene need further improvement.

6. CONCLUSION

In this study, we constructed two different CRISPR/Cas9 transformation vector which contain a sgRNA(s) to target NPT marker gene in the transgenic rice genome. We carefully designed these transformation vectors and then transformed the DH5α *E.coli* cells. Three colonies have been chosen and sent for sequencing for confirmation. The positive colonies which include CRISPR transformation vectors containing sgRNA(s) have been used for the bombardment of transgenic rice leaves or rice callus.

During transformation, we bombarded the leaves and callus two times. Then bombarded leaves or callus were incubated overnight at room temperature. After incubation leaves or callus were taken from bombardment medium and placed onto six-well plate.

GUS staining has been performed by using a commercial kit (Sigma). However, even after four days, no blue dot has been observed. This indicated that our transformation vectors were not sufficient to excise NPT gene from the transgenic rice genome. Also, we observed very few blue dots when we bombarded pCAMBIA1301 plant expression vector. The pCAMBIA1301 vector contains GUS gene and after successful transformation and following GUS staining, blue dots must have been seen. This situation suggests that transformation procedure also need to be optimized.

In conclusion, even though the plasmid we used has been used succesfuly to excise GUS gene before, in our case we were not able to excise NPT antibiotic resistant gene from transgenic rice genome. After sequencing the target site of putative transformed callus DNAs it will be easy to deduce about efficiency of CRISPR/Cas9 technology.

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