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***Full Length Article***

# Development of Founder Lines for Recombinase based Gene Targeting in *Nicotiana banthamiana*

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## Abstract

In plants, currently prevailing random transformation methods yield unpredictable results ranging from gene silencing to lethal effects. To address such adverse effects, site specific gene targeting has been on the wish list of biologists since the advent of genetically modified organisms. Site specific gene targeting and stacking is an emerging technology for precise genome maneuvering and pyramiding of two or more genes of interests at specific genomic locations. Recombinase based genome modifications is one of the recent tools to edit genomes at will. For recombinase based gene engineering, recombinase recognition sites are needed to be present in the target genome to delete, add or stack genes on. We have developed a founder line for site specific gene integration in a model plant *Nicotiana banthamiana*. The gene cassette was designed to introduce recombination sites as tag sites. The construct was transformed into *N. benthamiana* to introduce PhiC31 integrase recombination site (attP57) for subsequent transformation events. Cre recombinase LoxP sites were introduced in founder construct for marker gene removal allowing clean/marker free *N. banthamiana* plants. This recombinase-based founder line can be used as base line for subsequent transformation events at specific site. © 2019 Friends Science Publishers

**Keywords:** *Agrobacterium* mediated transformation; *Nicotiana banthamiana*; Recombinases; Site-specific gene integration

## Introduction

Plant transformation has become a routine technique of modern plant biology for improving genetic architecture and productivity of crop plants (Moeller and Wang, 2008). By increasing productivity of crop plants and lowering the cost to produce superior quality foods, advanced breeding procedures and genetic engineering tools have played a key role in food security and poverty alleviation. Adoption of these advancements is undoubtedly critical to keep feeding the increasing population of the world (Wang *et al*., 2010).

Plant transformation is achieved by two most commonly used methods; particle bombardment and *Agrobacterium* mediated transformation. Both methods result integration of genes at random locations within the genome with multiple copy transgenes and unpredictable transgene expression. Such genetic modification may lead to expensive and labor-intensive procedures to separate stable and single copy transgene insertions (Ow, 2005). During the last few decades, research efforts aimed to avoid random and multiple copy transgene integrations to eliminate unpredictable transgenic events (Ow, 2005). Use of site specific recombinases has emerged as a reliable technology to cope with the problems of random and multi copy transgene insertions. This technology has the ability to integrate gene of interest at specific site leading to predictable expression (Wang *et al*., 2010). Recombinases are the enzymes of bacteria and fungi having ability to catalyze DNA recombination at specific sites. These enzymes catalyze DNA recombination similar to recombination during the Pachytene stage of meiosis-I where homology-based exchange of segments of homologous chromosomes occurs between two non-sister chromatids. Genetic recombination results in new arrangement of alleles on homologous chromosomes (Snustad and Simmons, 2008).

Depending on the type and recombination sites, recombinases can cause DNA strand breakage, exchange of segments between homologous chromosomes and rejoining of DNA segments by the help of DNA ligases. Any double strand break in DNA triggers organism's indigenous repair mechanisms *i.e*., non-homologous end joining (NHEJ) and homology directed repairing (HDR) which are exploited for manipulating genome architecture. There are different types of recombinases having specific recombination sites. These sites are normally 30–40 nucleotides long. The outcome of recombination (Insertion, Deletion or translocation) of each recombinase is determined by the orientation of DNA recognition site (Grindley *et al*., 2006).

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Recombinase super family is divided into Tyrosine (Tyr) and Serine (Ser) recombinases. These groups are further subdivided on the basis of active amino acids located within the catalytic domain of each recombinase of the family. Both groups vary on the basis of mode of action and size of recombinases. Tyrosine recombinases are broadly subdivided into bidirectional tyrosine recombinases (Cre-LoxP, FLP-FRT and R-RS) (Wang *et al*., 2010) and unidirectional tyrosine recombinases (Lambda, HK101-1 and pSAM2) (Wang *et al*., 2010). Serine recombinases, also known as resolvases, are subdivided into small Serine recombinases (β-six (Diaz *et al*., 2001), CinH-RS2 (Kholodii, 2001; Thomson and Ow, 2006), γδ and ParA-MRS (Gerlitz *et al*., 1990; Thomson *et al*., 2009) and large Serine recombinases (Bxb1-att (Kim *et al*., 2003; Thomson and Ow, 2006), PhiC31-att (Thomason *et al*., 2001; Rubtsova *et al*., 2008), TP901-att (Stoll *et al*., 2002). These recombinases can catalyze deletion, integration, inversion and cassette exchange (Garcia-Otin and Guillou, 2006; Dymecki and Kim, 2007; Luan and White, 2007; Nern *et al*., 2011; Fenno *et al*., 2014).

Tyrosine group of recombinases work with their respective single DNA recognition sites. Cre recombinase works with LoxP site and FLP recombinase works with its FRT site only (Wang *et al*., 2010). Serine group of recombinases work with two DNA recognition sites that vary in sequence, *i.e*., attB (attachment site for bacteria) and attP (attachment site for phage). These enzymes recombine an attB site with an attP site causing deletion, insertion and inversion. The product sites are two irreversible hybrid sites attL and attR. These sites can be made reversible by the action of another enzyme, excisionase (Thorpe *et al*., 2000; Ghosh *et al*., 2006).

Site specific recombination systems assist bacteria and fungi in numerous molecular functions concerning viral phase turn over and insertion of phage gene to host genome. Integration, removal or inversion of DNA fragment could occur without loss or gain of nucleotides. Site specific recombinases recognize respective recognition sequence and catalyze DNA cleavage and reunion between complementary recombinase recognition sequences (Grindley *et al*., 2006). *N. banthamiana* has been a favorite plant model for biologists especially virologists due to hyper-susceptibility for a number of plant viruses infecting a wide range of crops. Not only viruses, it provides a subject for studying other plant processes like gene expression, regulation, cellular localization and plant host interactions (Goodin *et al*., 2008).

*N. banthamiana* has been extensively used by researchers soon after the advent of recombinant DNA technologies. Ability to express foreign genes from a bacteriophage was a major breakthrough. This advancement allowed study of viral trafficking in living tissues in addition to understanding plasmodesmetal gateways and macromolecular drive within and between cells. It opened new horizons for understanding protein targeting (Chapman *et al*., 1992; Cruz *et al*., 1996; Escobar *et al*., 2003; Lucas, 2006). Introduction of virus-induced gene silencing (VIGS) further made *N. banthamiana* a potential organism for understanding reverse genetics (Liu *et al*., 2002; Dong *et al*., 2007) frequently used to down regulate foreign genes in plant genome (Thomas *et al*., 2001). Comparatively, viral vectors used for VIGS were more workable in *N. banthamiana* than *Arabidopsis thaliana* (Deleris *et al*., 2006; Burch-Smith *et al*., 2007). The simple to use technique of Agro-infiltration resulted in more popularization of *N. banthamiana* as a model plant allowing transient expression of transgenes fused with a scorable marker for visible detection of expressed proteins (Goodin *et al*., 2002; Voinnet *et al*., 2003).

Recognizing the potential of *Nicotiana banthamiana* as a model plant, we designed this research for developing recombinase-based founder line. The resulting *Nicotiana banthamiana* founder line can serve as a base line for integrating genes of interest at the pre-determined genomic site in *Nicotiana banthamiana* genome to achieve gene stacking.

## Materials and Methods

*Agrobacterium* mediated transformation of *N. banthamiana* was conducted at Abhaya M. Dandekar Lab (AMD), Department of Plant Sciences, University of California, Davis, CA, USA.

### Plasmid Design

In the present study, we designed founder construct for using Cre recombinase and PhiC31 integrase. Cre recombinase can be used for marker gene removal if marker gene is flanked by two LoxP sites in same direction. PhiC31 integrase will be used by introducing attP57 sites in founder construct for further gene integrations. The attP site in founder line will be exploited by designing attB site in incoming exchange vector for integrating gene of interest (GOI). In each transformation event, this process moves on with integration of GOI and removal of marker gene simultaneously. The pG-Rec plasmid (4.6 kb) harboring NptII gene flanked between two loxP sites was excised from pJet-Rec and cloned at BglII and KpnI sites of pGreen-0029. Plasmid was transformed in *E. coli* cells and isolated to transform into *Agrobacterium* competent cells for plant transformation.

Founder vector aimed to carry specific DNA recognition sites to be integrated into plant genome (*N. banthamiana*). Founder vector was designed to assemble attP57 site for PhiC31 integrase in close proximity to loxP sites of Cre recombinase flanking the marker gene. NptII gene was designed to be transcribed by NOS-promoter. The whole assembly was labelled as Rec cassette. Plasmid pJet provided the backbone to form construct as pJet-Rec. Rec cassette was built in pJet between two restriction sites BglII and KpnI.

The pJet-Rec and pGreen-0029 vectors were restricted with BglII and KpnI to excise Rec cassette from pJet-Rec and ligation into pGreen-0029. NptII gene in pGreen-0029 attached to its promoter and terminator was replaced with Rec cassette to produce final vector as pG-Rec. Standard cloning procedures were performed according to Green and Sambrook (2012). The plasmid expression construct was transferred to *Agrobacterium* GV3101 strain through electroporation.

**Agrobacterium Mediated Transformation of *Nicotiana benthamiana***

Seeds of *N. banthamiana* were obtained from lot # 07090 from Plant Transformation Facility, University of California, Davis, CA, USA.

**Surface Sterilization of Seeds**

Seeds of *N. banthamiana* were transferred into 15 mL conical centrifuge tube for surface sterilization and immersed in sterilization solution (10% Clorox with 2–3 drops of Tween-20) for 10–15 min. Seeds were rinsed with sterile milli.Q.$H_2O$ for three times. Seeds were dried with sterile filter paper and were placed on 1/2MS-agar plates (2.215 g/L Murashige and Skoog minimal organics medium ($MS_o$) modified with 15 g/L Sucrose and 1.5% agar (pH 5.6-5.8). Plates were incubated at 26ºC. Emerging shoot tips were excised and sub-cultured to fresh medium after every 21 days.

***Agrobacterium* Infection**

Plasmid pG-Rec was transformed into *Agrobacterium* GV3101 strain and streaked on LB-Rifampicin and Gentamycin plate. Single bacterial colony was picked from freshly streaked plate and inoculated into 20 mL of MGL medium (5 g/L Tryptone, 5 g/L Sodium Chloride, 0.1 g/L Magnesium Sulphate, 0.25 g/L Potassium di-hydrogen phosphate and 1 g/L Glycine; pH 7.0) with kanamycin as an antibiotic. Bacterial culture was incubated overnight at 250 rpm at 28ºC. A 5 mL of overnight grown bacterial culture was taken and transferred to 15 mL of TY medium (6 g/L Tryptone, 3 g/L Yeast Extract and 0.38 g/L $CaCl_2$; pH 5.5) carrying Rifampicin and Gentamycin for GV301 cells and Kanamycin for pG-Rec plasmid selection in addition to 200 $\mu$M of Acetosyringone. Bacterial culture was again incubated at 28ºC for overnight at 250 rpm. Next morning, a 1.5 mL of overnight grown bacterial culture was transferred to 20 mL of TY medium of same concentration and pH carrying 200 $\mu$M of Acetosyringone and grown until the $OD_{600}$ of *Agro*-pG-Rec culture was achieved.

**Co-cultivation**

The newly expanded leaves were removed from *in vitro* plants and cut into 1 $cm^2$ leaf slices, while suspending in *Agro*-pG-Rec culture. The leaf slices were allowed to soak in the *Agro*-pG-Rec solution for 10 min. The leaf slices were placed abaxial side down on co-cultivation medium (4.33 g/L Murashige and Skoog minimal organics medium

**Table 1:** PCR Primers designed for confirmation of Rec cassette and NptII gene

| Name of Oligos | 5′→3′ Sequence | Purpose |
|---|---|---|
| NptII For. | AGTCCCGCTCAGAACAACTC | NptII gene |
| NptII Rev. | GTTGTCACTGAAGCGGGAAG | amplification 557 bp |
| Rec For. | GACCCTACGCCCCCAACT | Rec cassette |
| Rec Rev. | TCGTCGTCGACAAGCCGGATAA | confirmation 1493 bp |

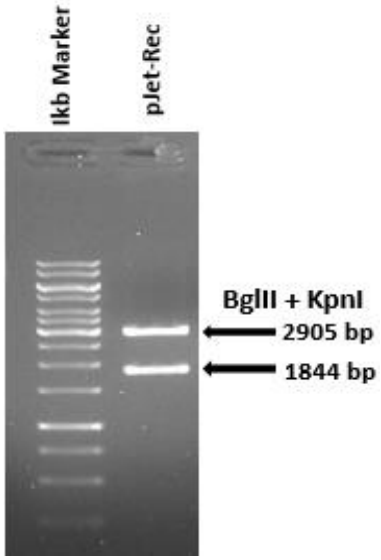


**Fig. 1:** Isolation of Rec cassette (1844 bp) from pJet-Rec by digestion with BglII and KpnI restriction enzymes and incubating digestion reaction at 37°C for 2 h

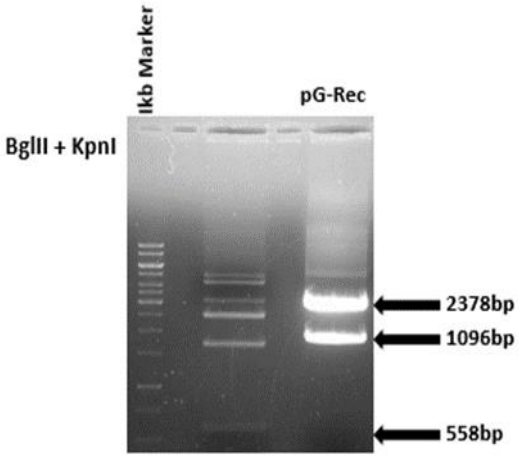


**Fig. 2:** NptII gene excision (1096 bp) from pGreen-0029 with BglII and KpnI restriction enzymes to replace it with Rec cassette (1844 bp) excised from pJet-Rec

($MS_o$) modified with 30 g/L Sucrose, 2.0 mg/L BAP and 200 $\mu$M Acetosyringone, pH 5.6–5.8). Leaf slices were incubated at 23°C in the dark for 2–3 days.

**Induction**

The leaf slices were transferred to induction medium ($MS_o$ modified with 30 g/L Sucrose, 2.0 mg/L BAP, 400 mg/L Carbenicillin, 250 mg/L Cefotexime and 100 mg/L Kanamycin sulfate, pH 5.6–5.8) after 2–3 days. Leaf slices were incubated at 26ºC for 10 days. They were sub-cultured to fresh medium of the same formulation after every 21 days until tiny buds appear.

**Elongation**

Tiny shoots were transferred to elongation medium ($MS_o$ modified with 30 g/L Sucrose, 0.1 mg/L Benzyl Amino Purine, 400 mg/L Carbenicillin, 250 mg/L Cefotexime and 100 mg/L Kanamycin; for pG-Rec selection, pH 5.6–5.8). *N. banthamiana* leaves were incubated at 26ºC for 10 days. They were sub-cultured to fresh medium of the same formulation after every 21 days until shoots form.

**Rooting**

On reaching 2–4 cm in height, shoots were harvested, labelled and transferred to rooting medium (1/2x MS medium modified 15 g/L Sucrose, 0.2 mg/L Indole Butyric acid, 400 mg/L Carbenicillin, 250 mg/L Cefotexime, 8 g Phytoagar and 100 mg/L Kanamycin, pH 5.6–5.8). Shoots started rooting within 14 days.

**Hardening**

On growing enough roots and shoots, *N. banthamiana* plants were allowed to harden within jars for a week. Lids of jars were opened for a brief period of time on first day of hardening. Time of opening jars was extended each day and they were allowed to grow in media without lids. Plants were shifted to sunshine mix in pots at controlled environment facility at UC-Davis. Initially plants were covered with a doom and were grown under controlled temperature.

**Maturity**

Plants were shifted to green house and were further allowed to grow till maturity. Plants started anthesis after 48 days of shifting to green house. Pale white colored flowers persisted for 3–4 days and started turning to pods after 21 days. Green pods contained small whitish seeds which turned to brown due to drying of *N. banthamiana* seeds. When pods started rupturing on turning brown they were collected for transgene analysis.

**Transgene Analysis**

DNA was extracted at mature leaf stage by cutting leaf slices from each plant. DNA extraction was done using QIAGEN DNeasy plant mini kit. DNA quantification ($OD_{260/280}$) was performed on a spectrophotometer. Two sets of primer pairs were designed for rec cassette integration confirmation within plant genome. The name of oligos used for each pG-Rec plasmid transformation event, their sequences and the gene to be amplified for transformation confirmation are given in Table 1.

## Results

**Restriction Analysis of Founder Clone**

Founder clone was constructed to carry Rec cassette (attP57 sites for PhiC31 integrase and loxP sites for Cre recombinase) to be transformed into *N. banthamiana* for the development of recombinase-based founder lines. pJet-Rec plasmid was digested to release 1844 bp and 2905 bp size DNA fragments. Gel purified fragment of size 1844 bp carrying NptII gene flanked between two loxP sites was retained for ligation with linearized pGreen-0029 (Fig. 1).

NptII gene of plasmid pGreen-0029 was replaced with loxP flanked NptII gene of pJet-Rec with BglII and KpnI. 1096bp restricted fragment of pGreen-0029, was removed through gel isolation (Fig. 2).

Rec cassette excised from pJet-Rec and linearized pGreen-0029 were ligated in the presence of T4 DNA ligase to obtain pG-Rec clone. The clone carried pGreen-0029 backbone and Rec cassette as fragment from pJet-Rec. The whole Rec cassette was concised between left and right border repeats in order to deliver T-DNA region to plant genome through *Agrobacterium* (Fig. 3).

BglII enzyme cut pG-Rec into two fragments of size 2453 bp and 2548 bp. KpnI linearized the vector to 5901 bp. Double digestion with both the enzymes together released three fragments of size 618 bp, 1835 bp and 3448 bp. All three digestions showed appropriate fragments for pG-Rec confirmation (Fig. 4). pG-Rec vector was constructed to be used as founder plasmid for developing recombinase-based founder line in *N. banthamiana.*

***Agrobacterium* Mediated Transformation of *Nicotiana banthamiana***

In the current work, *Agrobacterium* mediated transformation of *Nicotiana banthamiana* leaf disks has been employed for stable transformation and developing highly regenerable somatic calli. Regeneration process provided a good prospect to produce tiny plants in shortest possible time. To generate plantlets, explants were cultured on MS medium of different concentration for initiation of callus, elongation of buds and rooting of emerging leaves. The edges of leaf disks protruded soon

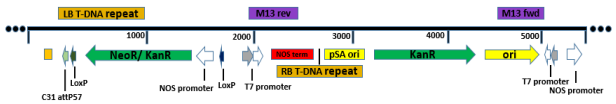


**Fig. 3:** pG-Rec (5901 bp) showing NeoR/KanR gene flanked between two LoxP sites for Cre recombinase and an attP57 site for PhiC31 integrase. Whole assembly was assembled between left (LB) and right boarder (RB) repeats of T-DNA. A KanR gene was placed outside the borders for bacterial selection

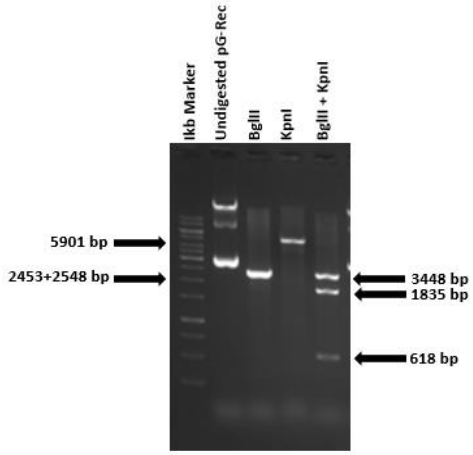


**Fig. 4:** pG-Rec (5901 bp) was confirmed by digesting plasmid showing three fragments as nicked, circular and super helical. Single digestion with BglII restriction enzyme gave two fragments (2453 and 2548 bp). Digestion with KpnI gave single fragment (5901 bp). Double digestion with BglII and KpnI released three fragments (3448, 1835 and 618 bp)

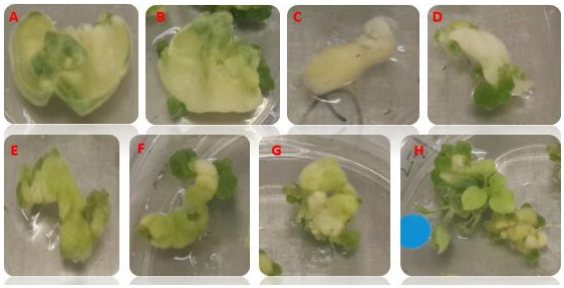


**Fig. 5:** *Agrobacterium* mediated transformation and regeneration of *Nicotiana banthamiana.* Leaf disk started curling from margins, immediately after transforming them with *Agrobacterium* culture and became pale yellow in appearance (A). Squeezed leaf disks developed tiny buds from the wounded ends and were distinguished from growing callus (B). Tiny buds were carefully excised and shifted to fresh medium prior of being masked by calli overgrowth (C). Buds started developing like normal leaf tissue (D) and regenerated calli covering entire bud area (E, F & G). Calli then developed tiny leaflets leading them to mature leaves (H)

after placing them to induction medium.

Induction of calli started after 1 week of incubation on medium of same formulation. Emerging calli initially developed tiny buds/leaflets and then originated true leaves. Regeneration process completed within 2–3 months after transformation (Fig. 5). Mature leaf disks were carried through *in vitro Agrobacterium* mediated transformation. Sensitivity to selection agent (antibiotic resistance) marked tiny plants as fertile transgenic *N. banthamiana*. For optimizing a suitable concentration of kanamycin to *N. banthamiana*, a kill curve test was done on un-transformed leaf disks under similar conditions to regeneration with different concentration of antibiotic on each plate. A negative correlation was observed between antibiotic concentration and leaf disks as well as bulging calli survival (data not shown). Lethal dose was selected for transformed calli selection. Leaf disks, bulging calli, protruding leaflets and their development to mature plant were all carefully selected at lethal dose (Fig. 6). Mature leaves and seeds were harvested for further subjection to transgene analysis.

### Transgene Analysis

Extracted DNA samples of leaf disks excised from mature transformed leaves were subjected to PCR with Rec primers for confirmation of Rec cassette integration into *N. banthamiana* genome. PCR of extracted samples showed expected fragments of size 1493 bp in almost 80% of the tested leaves (Fig. 7a). Extracted DNA was further subjected to PCR analysis with NptII gene specific primers for identifying the presence of Kanamycin gene within plant genome. PCR amplified a 557 bp fragment in all samples already confirmed with Rec cassette primers. These primers amplified NptII gene located within loxP sites of Rec cassette (Fig. 7b).

## Discussion

A major drawback in advancement of plant genome engineering research is difficulty in manipulating multiple genes. Numerous gene stacking techniques are available for pyramiding genes in plants. Most of them are based on random integration of multiple transgene (Halpin, 2005). Integration of foreign genes site specifically is genome friendly and produce predictable results. Incorporation of novel traits to prevailing cultivars without disrupting number of loci may be possible through integration of foreign genes site specifically. This can bypass breeding procedures to incorporate genes by sequential crossing and backcrossing. Introducing a strategy for marker gene removal may further strengthen the scheme to produce clean/marker free plants (Hou *et al*., 2014).

Crops of different ecosystem need to be stacked with multiple genes to cope for maintaining sustainable agriculture. Several genome editing tools have been identified during last two decades that are based on site specific DNA recombination (*Cis* and *Trans*) by introducing recombination sites within plant genome.

The precision and affectivity of numerous DNA recombinases made them a method of choice for plant

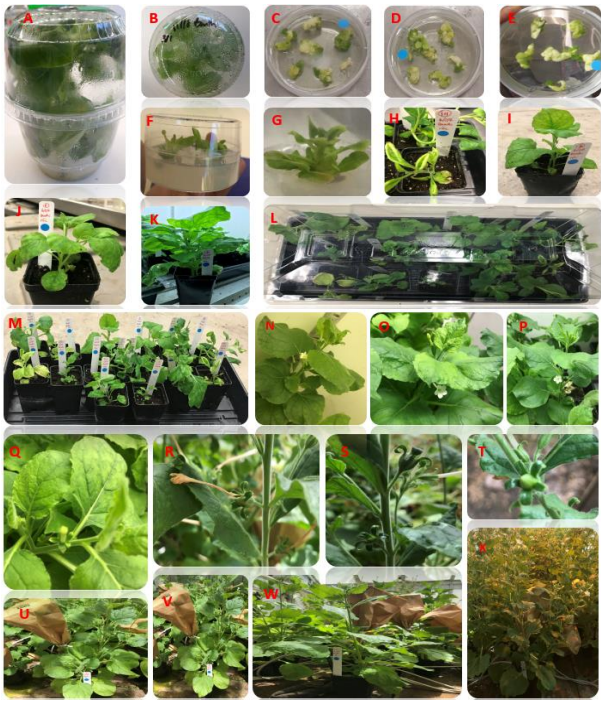

**Fig. 6:** Development of transformed *Nicotiana banthamiana. In vitro* plants were grown from sterile mature seeds of *N. banthamiana* in MS medium (A & B). Leaf disks were excised from these *In-vitro* plants and were infected with *Agro*-pG-Rec culture. Infected disks were grown on co-cultivation medium (C). Calli were induced from leaf disks by placing them on induction medium. Calli were cultured and sub-cultured on same induction medium for buds and leaf initiation (D & E). Tiny plants started developing roots while sitting on rooting medium in jars. Plants were allowed to grow within same jar till they grew to cover complete jar (F). Plants were hardened on developing 8-10 leaves by opening lids of jars (G). They were shifted to pots filled with sunshine mix at controlled temperature and were immediately covered with a doom to rescue them from drying (H). Potted plants were grown for a week to acclimatize shift from medium to soil (I, J & K). Plants were still grown under doom for further 3-4 days to prepare them for green house conditions (L). They were gradually transferred to open air environment in green house (M). Plants were allowed to grow in pots for 2-3 months for anthesis (N). Plants started producing white colored flowers (O & P) and developed pods after 28-30 days (Q). They were covered with paper bags to avoid cross pollination. Petals started wilting (R) exposing green colored tiny pods carrying whitish immature seeds (S & T). Pods were remained covered with bags to avoid any contamination and identification of self-pollinated seeds (U, V & W). Mature pods shed brown colored seeds for taking them to next progeny. Seeds were harvested from tagged pods (X)

genome engineering. These recombinases are used primarily for deleting selectable and scorable marker genes, once transformation is complete, with numerous applications like additions or exchanges.

Tyrosine group of recombinases are reversible in their action and cannot be used for gene stacking purpose

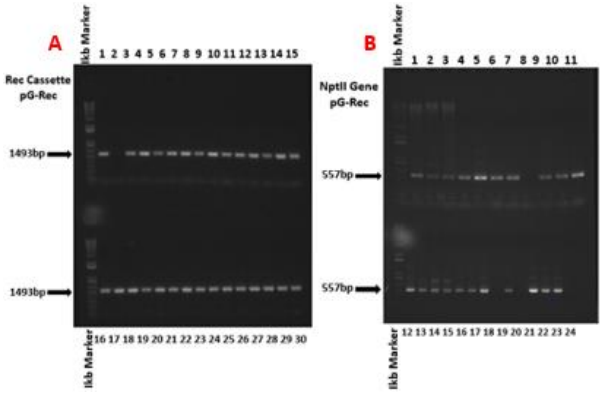


**Fig. 7:** Rec cassette confirmation in *Agrobacterium* mediated transformed plants by Rec primers (1493 bp) shown in A and NptII primers (557 bp) shown in B

involving multiple transformation events. Major advancement was brought by the use of irreversible DNA recombinases with ability to add or exchange foreign DNA molecules. Gene stacking has emerged as a potential approach for targeting agriculturally important crops which are less prone to transform with conventional molecular biological procedures (Srivastava and Thomson, 2016).

The major aim of the current piece of work was to develop a target line for site-specific integration of genes. We successfully developed a recombinase-based founder line for site specific genetic modification of *N. banthamiana*. Site specific gene insertion will be advantageous in selecting *N. banthamiana* lines for precise and stable transgene with single copy insertion without disrupting adjacent genes. *N. banthamiana* founder line has loxP sites for the removal of unnecessary DNA sequence *i.e.,* selectable marker gene, rendering *N. banthamiana* plants as a marker free. Developed founder line will save time and money for initial random transgene insertion and then selecting for most precise lines by breeding approaches, as founder line contains attP sites for restricting transgene integration at specific site for each subsequent transformation event.

Recombinases are site-specific DNA scissors working on cell's inherent DNA repair system during integration/excision, without gain or loss of nucleotides (Wang *et al*., 2010). Founder vector was designed to carry sites for two recombinase enzyme systems; one for marker gene removal and other for GOI integration. Recombinases recognize specific DNA recognition sites as Cre work with loxP sites for marker gene removal and PhiC31 works with att sites for gene integration. On standard basis, site specific gene stacking took place due to sequential transformation events of alternating an attP and attB recombination system with first transformation event as random (Ow, 2003). Rec cassette integration event was random carrying attP57 site for PhiC31 integrase and loxP sites for Cre recombinase. This random event has specified the site for further

transformation events that will be conducted for gene editing and stacking purposes. Integrating attP57 sites into *N. banthamiana* genome marked a specific site to enable further transformations to be site specific. Hence, we named the line produced through plasmid pG-Rec transformation into *N. banthamiana* as "Founder line". This line will serve as standard base line for recombinase-based gene removal, integrations and cassette exchange. Similar founder lines have been developed in citrus for initiating recombinase mediated cassette exchange. Citrus transgenic "Carrizo" was developed using same strategy for having resistance against Huanglongbing (HBL) disease and combating with *phytophethora* (Thomson, 2013).

Stable rice transgenic lines expressing GusA reporter were developed by Cre-LoxP recombination system (Srivastava *et al*., 2004; Chawla *et al*., 2006). Similar lines were also developed in *Drosophila melanogaster* using two different recombination systems for integration. Efficient gene integration by Bxb1 and PhiC31was utilized for site specific gene stacking in *Drosophila melanogaster* (Huang *et al*., 2011).

A unique strategy for gene integration/removal was employed for centromeric DNA fragment assembly in minichromosomes of mammals by combining Cre-LoxP and PhiC31-att system (Dafhnis-Calas *et al*., 2005; Xu *et al*., 2008). By engineering recombination sites, it is possible to use Cre as gene addition system (Day *et al*., 2000) and PhiC31 as gene deletion system (Thomson *et al*., 2012). In another report, PhiC31-att and Bxb1-att system were together used for gene integration along with Cre-LoxP system for marker gene removal in tobacco (Hou *et al*., 2014). Cre-LoxP mediated marker gene removal made the basis of commercial high lysine content corn (Ow, 2007).

## Conclusion

In our study, we successfully developed a recombinase-based founder line for gene targeting in *N. banthamiana.* Multiple genes of interests could be stacked at the specific site within founder line. This founder line also allows recycling the marker gene for multiple transformation events.

### Future Prospects

Initial founder line has been developed in *N. banthamiana* due to its importance as a model system and accuracy of transformation. We are utilizing this founder lines for site specific gene staking against viral diseases like cotton leaf curl virus (CLCuV), a serious threat to cotton production in Pakistan. Integration of multiple genes at specific genomic location against CLCuV will result durable resistance to the disease, as CLCuV has been reported to recombine rapidly to evolve new strains which causes breakdown of plant resistance.

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