Developing genetic elements including DNA promoters and vectors to modify plant genome including tobacco using CRISPR-Cas9-sgRNA genome editing system

Indu B. Maiti[1], Ph.D. , Dipak K. Sahoo[1], Ph.D. and Nrisingha Dey[2], Ph.D.

[1]*KTRDC,* , College of Agriculture-Food and Environment, University of Kentucky, Lexington, Kentucky 40546, USA; [2]Department of Gene Function and Regulation, ILS, Department of Biotechnology, Government of India, Nalco Square, Chandrasekherpur, Bhubaneswar, Orissa, India.

2014 TSRC, September 28-October, 2014,
Charlottesville, VA, USA

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf

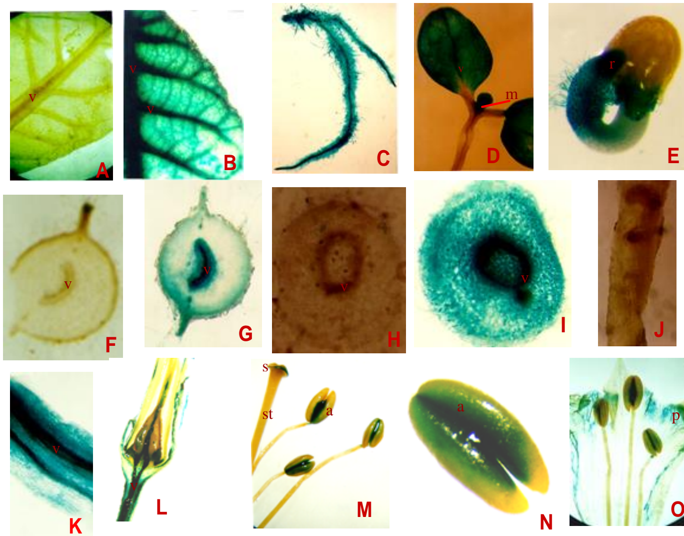
TSRC2014(68) - Document not peer-reviewed

Plant Genetic Engineering Research and Services

KTRDC, College of Agriculture, Food & Environment, University of Kentucky

Web: http://www.uky.edu/KTRDC/maiti-area.html

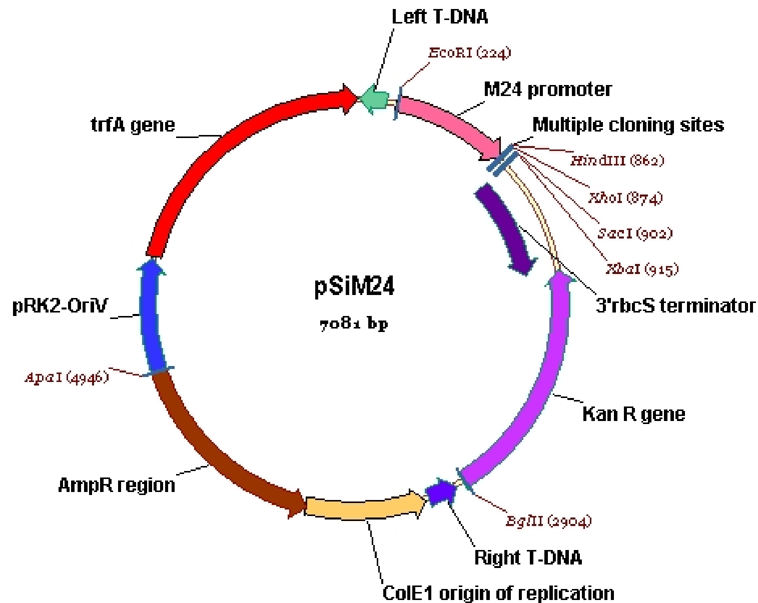
- **We created a number of engineered DNA promoter systems for enhanced expression of genes in transgenic plants. Analysis of these genetic promoters are published in referred journals.**



UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

A versatile binary vector pSiM24 for molecular biology and biotechnology applications



Physical map of pSiM24 , a modified binary vector, size 7081 bp, selection markers (KanR and AmpR genes). NCBI GenBank accession no. KF032933.1

Genetic components

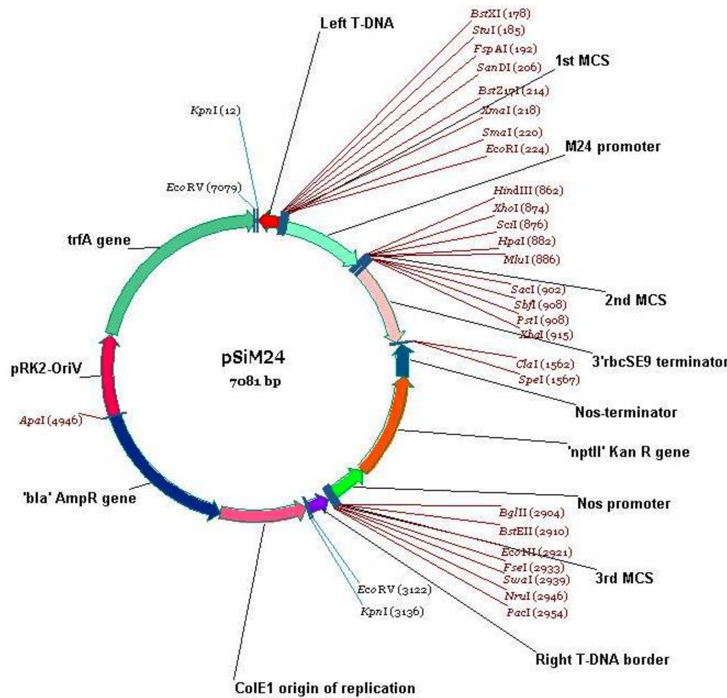
T-DNA portion

1. Left DNA (coordinates 13 to 169), left T-DNA border (65 to 90), complement.
2. M24 promoter (coordinates 223 to 860)
3. Multiple cloning sites: (861 to 914)
4. 3'rbcS terminator: (915 to 1565)
5. KanR gene, complement (1566 to 2911); KanR-terminator (1566 to 1820 complement); KanR-cDNA (1821-2618, complement); KanR-promoter (2619 - 2903, complement).
6. Right T-DNA (coordinates 2957 to 3118), right T-DNA border 3034 -3059, complement.

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

We have evaluated this new improved expression vector pSiM24 in transient assay and also stable expression experiments using reporter genes GFP and GUS



Schematic presentation of binary vector pSiM24 The backbone structure of binary vector pSiM24, **(7081-bp)** containing the modified full-length transcript promoter (M24) of the *Mirabilis mosaic virus* directs the coding sequences of the gene of interest, Left T-DNA and right T-DNA borders (Left T-DNA, Right T-DNA), selection marker genes (KanR, neomycin phosphotransferase II, nptII) directed by nopaline synthase promoter (Nos promoter), terminator sequences of ribulose bisphosphate carboxylase small subunits (3’rbcSE9), nopaline synthase terminator (Nos terminator), multiple cloning sites (**1st MCS, 2nd MCS and 3rd MCS**) having various restriction sites, replicon units **pRK2 oriV, trfA gene for Agrobacterium**, **ColE1 origin of replication for E. coli,** and ‘bla’ AmpR gene for resistance to ampicillin are depicted.

UNIVERSITY OF KENTUCKY

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

2014_TSRC84_Maiti.pdf

Comparative analysis of three binary vectors: pCAMBIA2300, pKM24KH (a derivative of pKYLX71) and pSiM24

(1) Transformation frequencies of *Escherichia coli* strain TB1 and *Agrobacterium tumefaciens* strain GV3850 for pSiM24 binary vector is about 5- and 2- folds more, compared to pCAMBIA2300 and pKM24KH, respectively.

(2) Binary Ti vectors pSiM24 produced higher plasmid DNA yields in *Escherichia coli* strain TB1 over pCAMBIA and pKM24KH, 4- and 8- fold, respectively.

(3) Plant transformation:

Binary Ti vectors pSiM24 and pSiM24-GUS/GFP conferred a kanamycin-resistant fresh weight (FW) increase in tobacco leaf discs after transformation with *A. tumefaciens*. No significant difference in fresh weight was noted for pSiM24, pCAMBIA and pKM24KH

(4) Effect of binary Ti vectors pSiM24 and pSiM24-GUS/GFP on transformation frequencies in *A. thaliana*; pSiM24 was more efficient (about 2 fold) compared to pCAMBIA and pKM24KH

UNIVERSITY OF KENTUCKY

TSRC2014(68) - Document not peer-reviewed

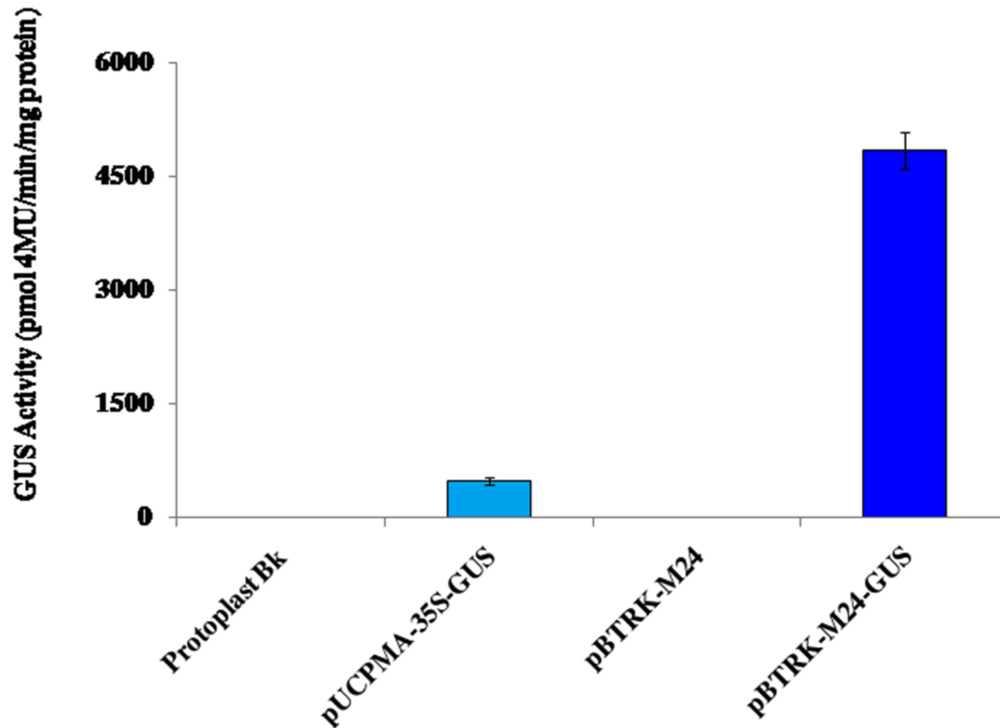
We have evaluated this new improved expression vector pSiM24 in transient assay and also stable expression experiments using reporter genes GFP and GUS, as well as real genes like GFP fused *Arabidopsis* CESA3$^{ixr1\text{-}2}$ (GFP-AtCESA3 $^{ixr1\text{-}2}$), vegetative insecticidal protein (Vip3A(a), Kinetoplastid membrane protein-11 (KMP-11), Interleukin 10 (IL-10) and native-T-Phylloplanin fused GFP (*nat-T-phyllo-GFP*).

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

The transient GUS expression analysis of T-DNA assembled fragment of pSiM24 in tobacco protoplast system

2014_TSRC84_Maiti.pdf

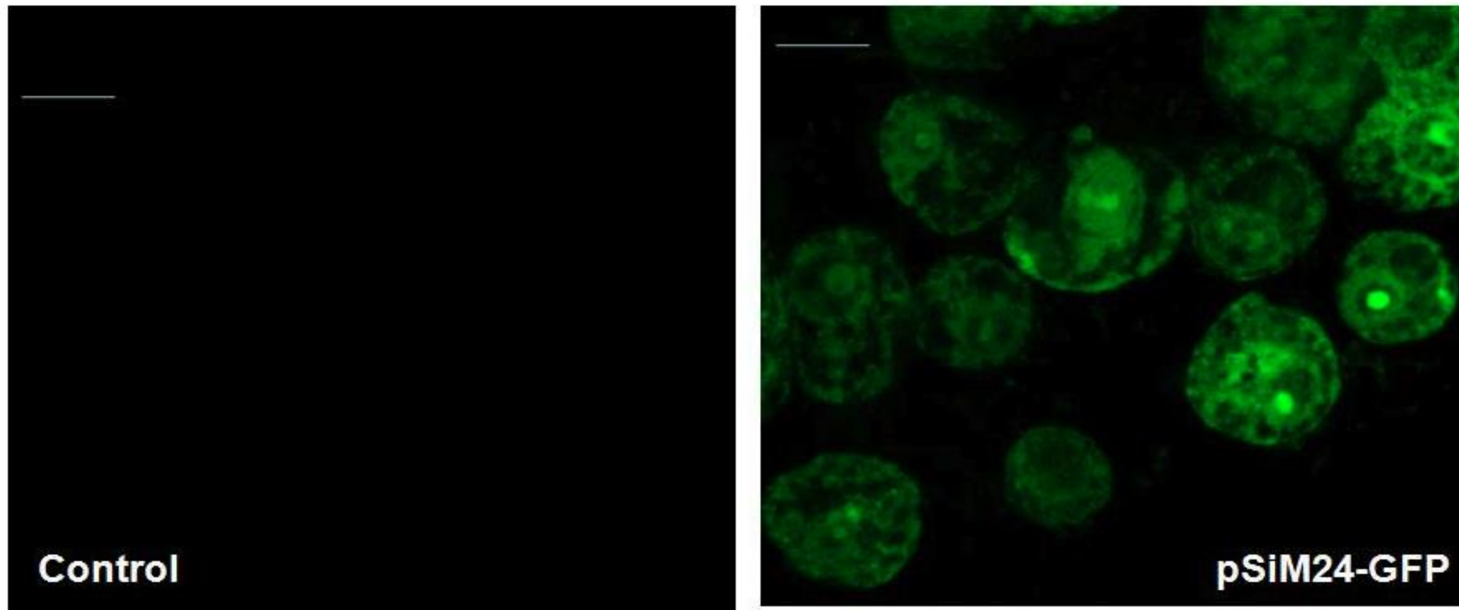


Transient GUS expression analysis of pBTRK-M24 (T-DNA assembled fragment of pSiM24), pBTRK-M24-GUS (pBTRK-M24 with GUS reporter gene) constructs in tobacco protoplast. The pUCPMA-35S-GUS construct carries the constitutive CaMV 35S promoter. The average GUS activity ± SD was presented in the histogram of 3 independent experiments of 3 replications of each construct. The values significantly differ between tobacco protoplasts with pBTRK-M24-GUS from others at $P < 0.01$ using Student's *t* test.

UNIVERSITY OF KENTUCKY

TSRC2014(68) - Document not peer-reviewed

Fig. 4. Transient expression of pSiM24-GFP in tobacco protoplasts.



Protoplasts were transfected with plasmids pSiM24 (Control) and pSiM24-GFP (having GFP reporter gene). Transformation efficiencies were determined by analyzing the protoplasts with fluorescence after incubation for overnight. Scale bar, 20 µm. Fluorescent confocal laser scanning micrographs of tobacco protoplasts are presented.

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

Fig. 5. The transient GUS expression analysis of pSiM24-GUS by *Agrobacterium* infiltration assay in *N. benthamiana* leaf, histochemical detection of GUS.

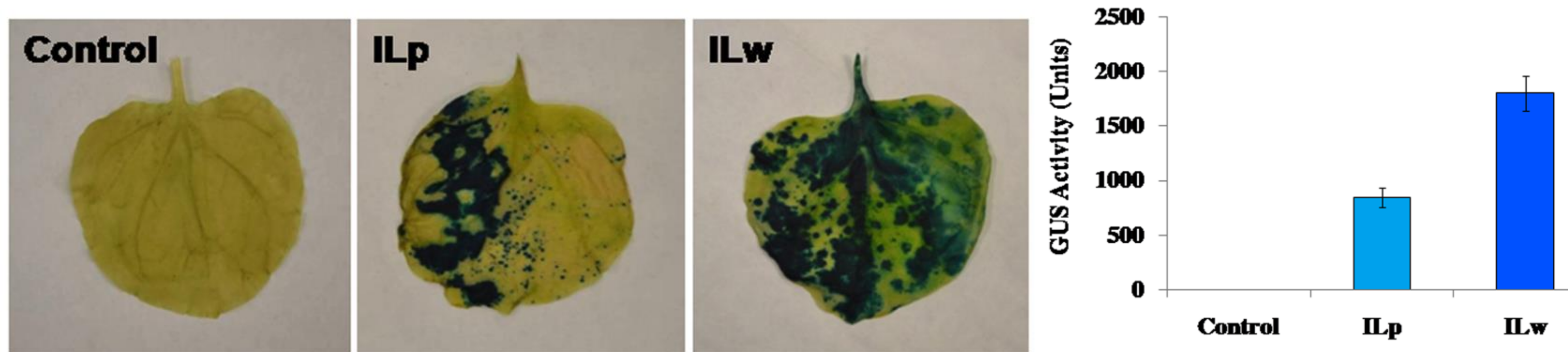
Representative transient GUS expressions by Agrobacterium infiltration assay in N. benthamiana leaves are shown for pKCaMV35S-GUS and pSiM24-GUS constructs. GUS was detected histochemically. Control represents for pSiM24 without GUS reporter gene.

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

2014_TSRC84_Maiti.pdf

Figure 11: Transient expression of GUS in pSiM24-GUS Agro-infiltrated *N. benthamiana* leaves using vacuum infiltration method



Control: N. benthamiana leaf infiltrated with A. tumefaciens harboring pSiM24 construct; ILp: Partial Infiltrated Leaf, ILW: Whole Infiltrated Leaf

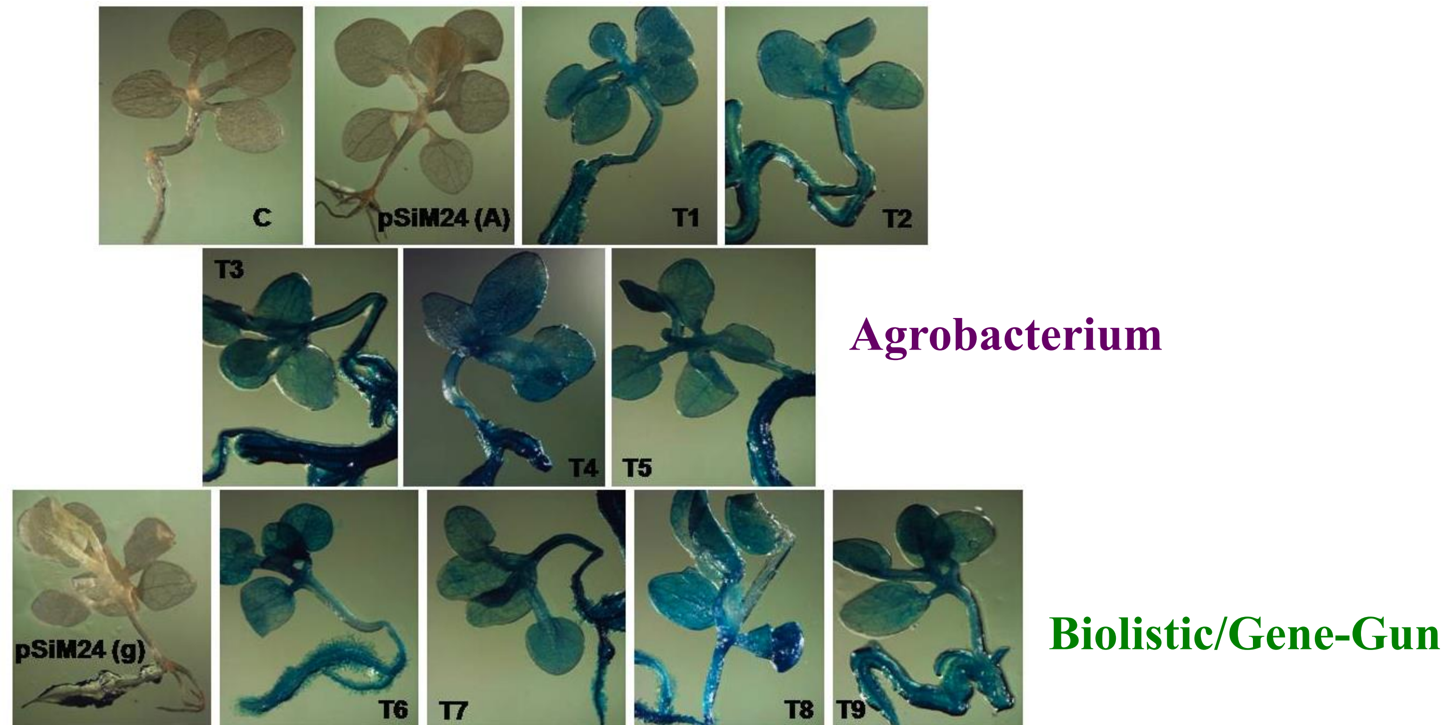
pSiM24 vector is useful for plant made product (PMP) applications

Reference: Sahoo DK, Dey N, Maiti IB (2014) pSiM24 Is a Novel Versatile Gene Expression Vector for Transient Assays As Well As Stable Expression of Foreign Genes in Plants. PLoS ONE 9(6): e98988. doi:10.1371/journal.pone.0098988

UNIVERSITY OF KENTUCKY

TSRC2014(68) - Document not peer-reviewed

Fig. 7. GUS expression in transgenic tobacco plants generated for constructs pSiM24 and pSiM24-GUS.



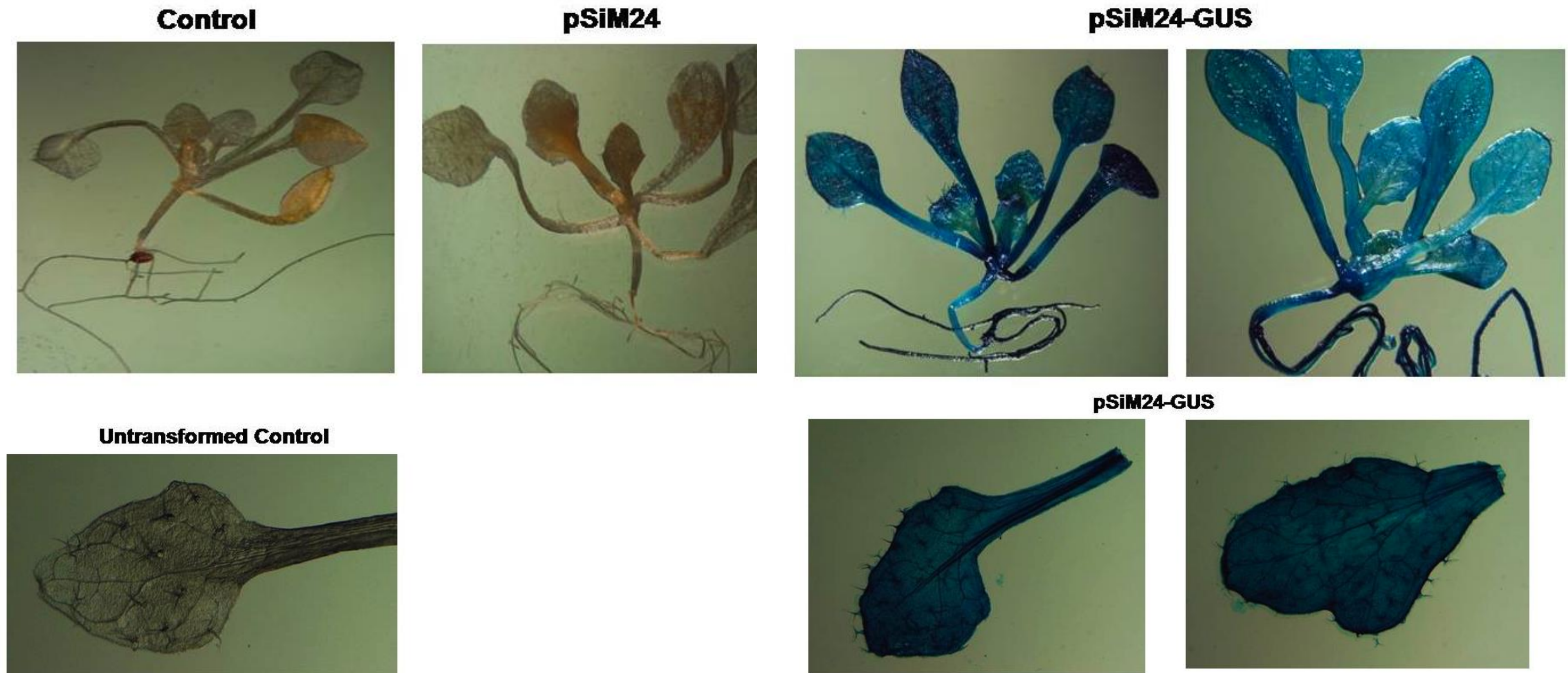
Representative transgenic tobacco plants (2nd generation, 3 weeks old) generated by agrobacterium mediated transformation (T1 to T5 lines) and transformation using gene-gun (T6 to T9 lines) were stained for GUS histochemical activity. C: Untransformed tobacco plant; pSiM24 (A): pSiM24 transgenic tobacco plants generated by agrobacterium mediated transformation; pSiM24 (g): pSiM24 transgenic tobacco plants generated by biolistic bombardment method.

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf

TSRC2014(68) - Document not peer-reviewed

Fig. 8. GUS expression in transgenic Arabidopsis plants generated for constructs pSiM24 and pSiM24-GUS.



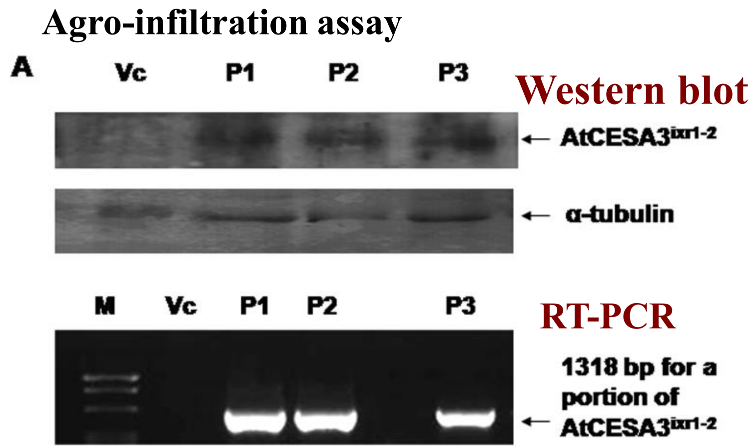
Representative transgenic Arabidopsis plants (2nd generation, 2 weeks old) generated by agrobacterium mediated transformation were stained for GUS histochemical activity. Control: Untransformed Arabidopsis plant; pSiM24: pSiM24 transgenic Arabidopsis plants; pSiM24-GUS: pSiM24-GUS transgenic Arabidopsis plants.

UNIVERSITY OF KENTUCKY

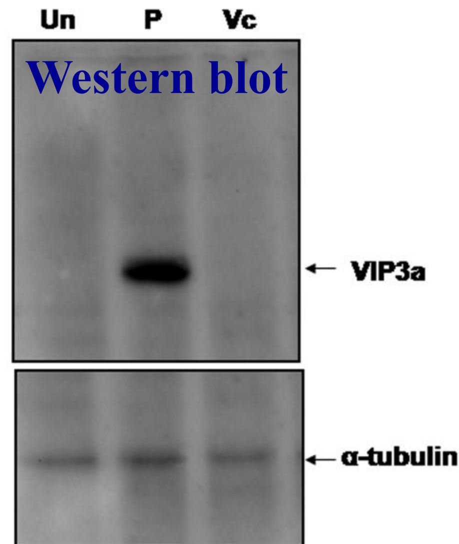
2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

Figure 13: Transient expression of GFP fused *Arabidopsis* CESA3^{ixr1-2} (GFP-AtCESA3^{ixr1-2}), vegetative insecticidal protein (Vip3A(a), Kinetoplastid membrane protein-11 (KMP-11) and Interleukin 10 (IL-10) using pSiM24 vector

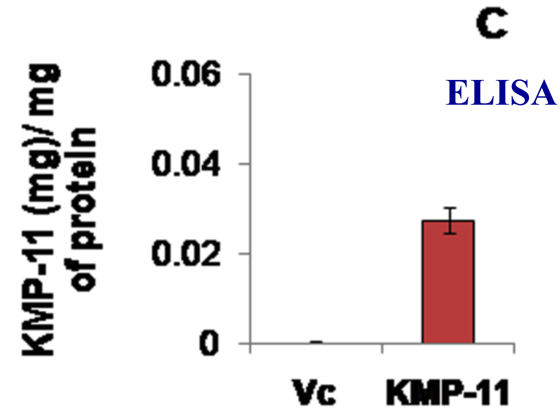
Agro-infiltration assay

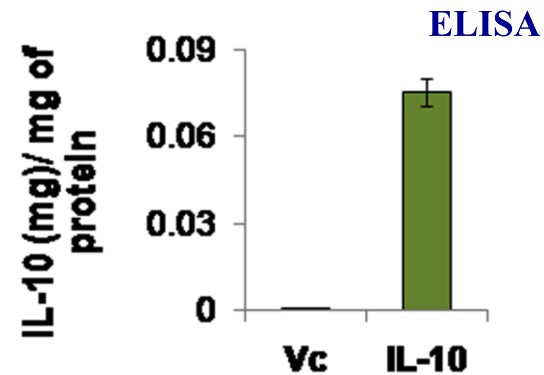


Tobacco protoplast



Tobacco protoplast



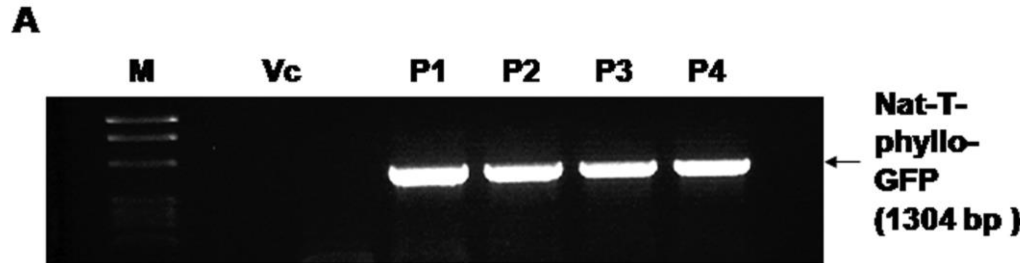


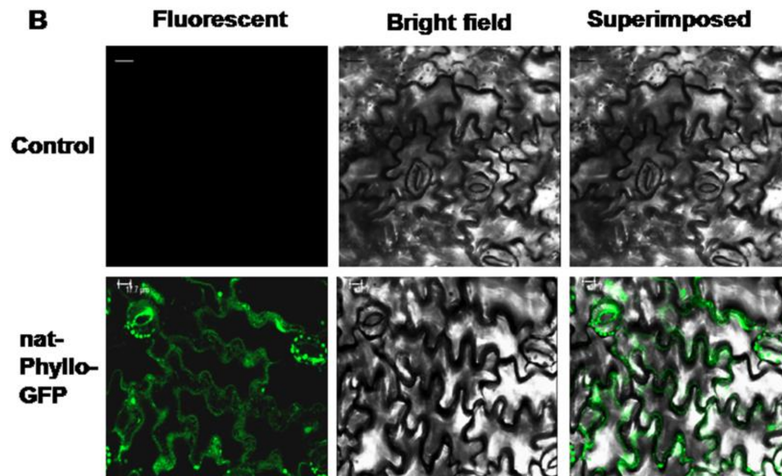
UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

Figure 14: Transient expression (Agro-infiltration) of fused nat-T-phylloplanin-GFP gene using pSiM24 vector

RT-PCR assay





Localization analysis of apoplast targeted nat-T-phyllo-GFP by confocal laser scanning microscope.

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

Genome Editing

What is CRISPR-Cas system

CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats

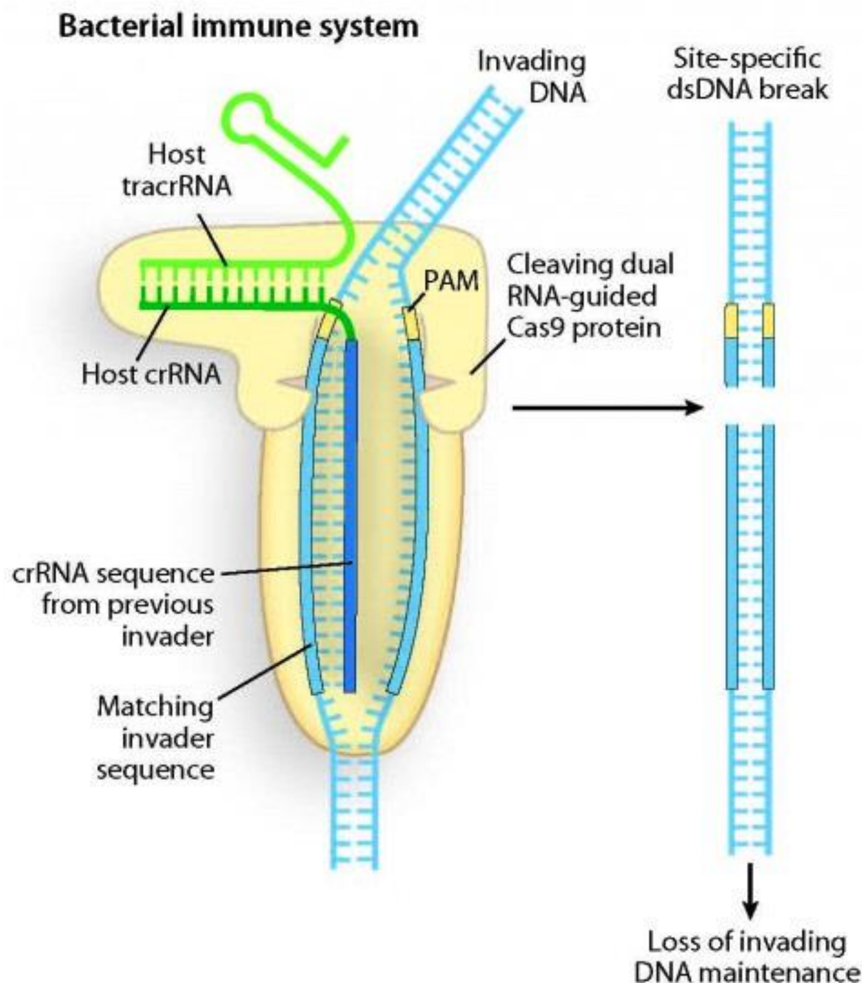
Arrays of regularly spaced DNA repeats present in archaea and bacteria

Some inervening repeat sequences (spacers) are acquired from identical DNA fragments (protospacer) in bacteriophases and plasmids

Cas (CRISPR associated) genes

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

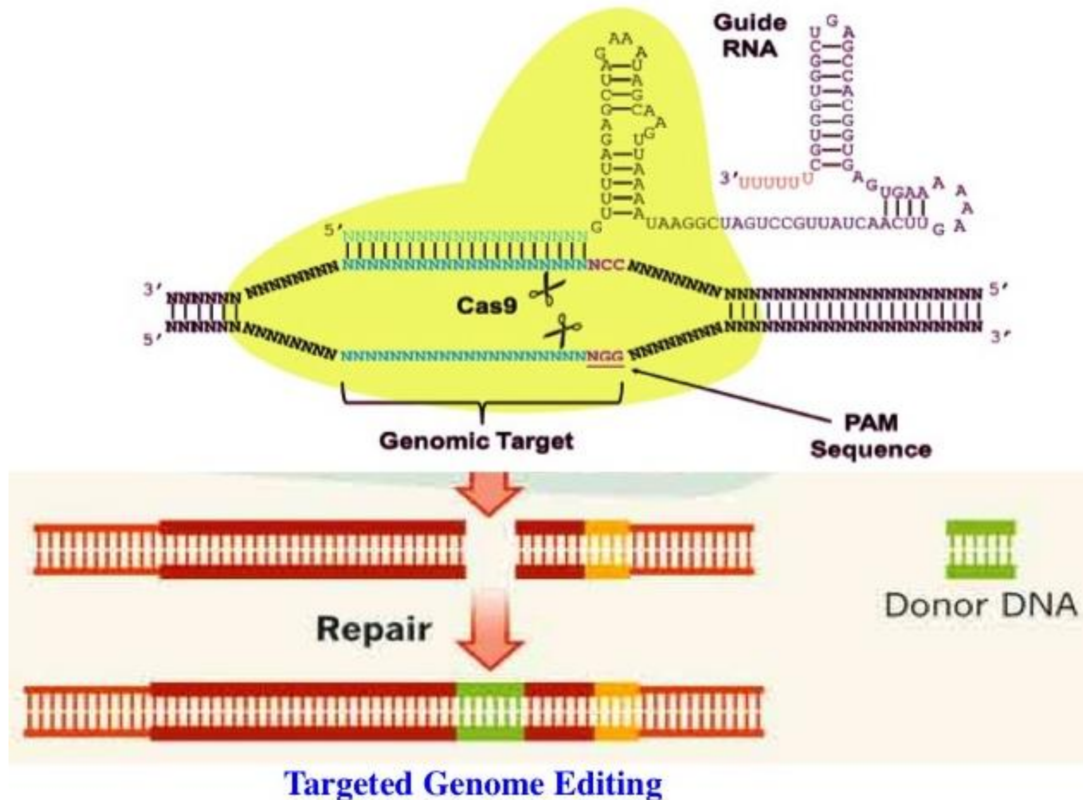
Archaea and Bacteria Immune System to Target Foreign Invader DNA



CRISPR and CRISPR-associated (Cas) system generates adaptive and heritable immunity against foreign genetic elements in archaea and bacteria

2014_TSRC84_Maiti.pdf

TSRC2014(68) - Document not peer-reviewed



A schematic of targeted gene disruption by Cas9/sgRNA complex and subsequent error-prone DNA repair by the nonhomologous end-joining (NHEJ). Cas9 separates DNA strands and it allows sgRNA to hybridize with a specific 20-nt sequence in targeted gene, it directs nuclease activity proximal, 3-nt upstream, to the protospacer-associated motif (PAM) site. Then the double stranded break is restored by nonhomologous end-joining (NHEJ) repair process in cells with donor sequence designed to insert or delete specific genomic sequence.

2014_TSRC84_Maiti.pdf

TSRC2014(68) - Document not peer-reviewed

Basic structure and organized domains of *Steptococcus pyogenes* Cas9 protein

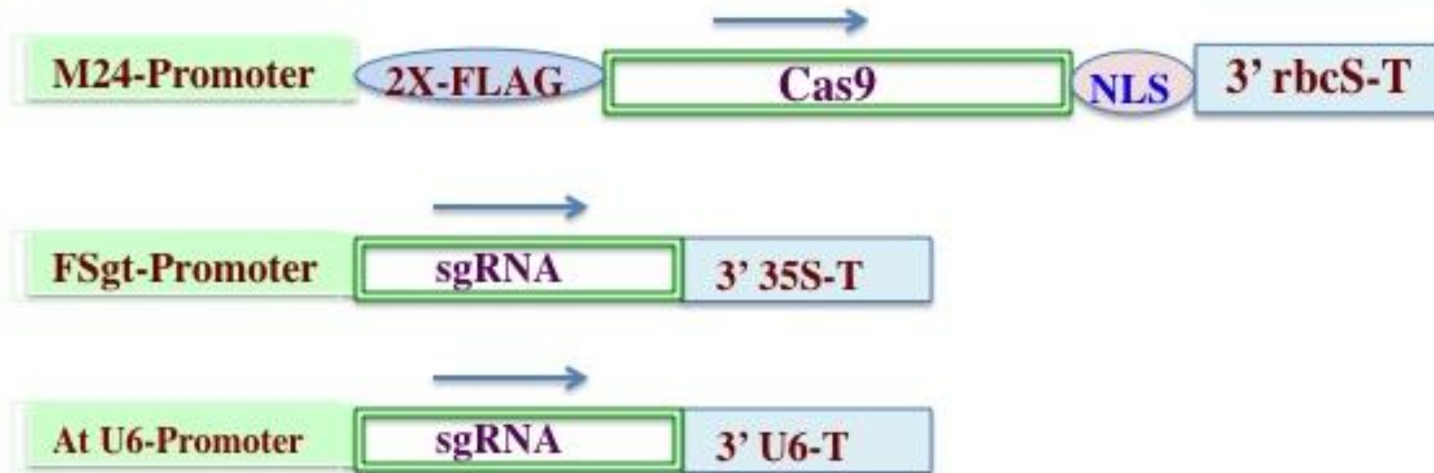
C
REC2 domain
REC1 domain
HNH domain
sgRNA
Tetraloop
Target DNA
Bridge helix
RuvC domain
PI domain
3'
5'
5'
3'
180°
Repeat:Anti-repeat duplex
Guide:Target heteroduplex
Stem loop 1
Linker
Disordered linker
Gly1366 (C)
Stem loop 2
Lys3 (N)
Stem loop 3

Crystal structure (ribon) of the Cas9-sgRNA-DNA complex

Nishimasu et al., Cell 156: 935-949, February 27, 2014

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

Physical map of Cas9 and sgRNA constructs designed for CRISPR-Cas9-mediated genome editing in plants including tobacco



Target genes:

(1) To minimize harmful carcinogen in tobacco, target nnd gene - reduce conversion of nicotine to nornicotine in tobacco plants.

(2) To develop genetic resistance against multiple plant virus pathogens, target translation initiation factor eIF4E gene.

(3) To develop male sterility, target male fertility genes.

(4) To reduce tobacco suckering, target sucker –producing genes.

2014_TSRC84_Maiti.pdf

TSRC2014(68) - Document not peer-reviewed

Summary

1. We have constructed a small and highly efficient binary Ti vector pSiM24 (GenBank Accession no. KF032933.1) for higher plant transformation. The size of the backbone of earlier binary vector pKYLXM24 (GenBank Accession No. HM036220), a derivative of pKYLX71 was reduced from 12.8 Kb to 7.1 kb.

2. The pSiM24 plasmid offers a wide selection of cloning sites, high copy numbers in *E. coli* and high cloning capacity for easily manipulation of different genetic elements.

3. It has been fully tested in transferring the transgene (GFP and GUS) in both transiently (agro-infiltration, protoplast electroporation and biolistic), and stably in plant systems (*Arabidopsis* and tobacco) using both agrobacterium-mediated transformation and biolistic procedures, hence this would be useful for both nuclear transformation, and plant made products (PMP) applications.

2014_TSRC84_Maiti.pdf
TSRC2014(68) - Document not peer-reviewed

4. For gene regulation, we have developed RNAi vector using pSiM24 backbone. For RNA-guided genome editing applications, we are in process of developing CRISPR-Cas9-sgRNA system using pSiM24 vector.

5. We have created a series of DNA promoters useful for expressing foreign genes in transgenic plants. Those promoter systems have been published in referred journals. We could make available those DNA promoters with our highly efficient Ti binary vector pSiM24 to scientific community in nation and abroad for evaluating in economically important various crop plants like soybean, tomato, maize, cotton, to name a few, and other plants like energy crops maize, poplar, switch grass.

http://www.ca.uky.edu/KTRDC

www.uky.edu/ktrdc/maiti-area.html

Thank you

UK UNIVERSITY OF KENTUCKY

UNIVERSITY OF KENTUCKY

2014_TSRC84_Maiti.pdf

TSRC2014(68) - Document not peer-reviewed