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CRISPR-Cas9-Mediated Editing of the *CYP82E4-Nicotine N-Demethylase (nnd)* Gene in Tobacco Protoplasts

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Abstract

The single RNA-guided DNA recognition CRISPR-Cas9 method is a simple and powerful tool for targeted genome engineering. Here, we report the designing and testing of efficient caulimoviral promoter-derived binary vectors for performing genome editing employing the CRISPR-Cas9-gRNA system. For such targeted mutagenesis, we created binary transformation vectors to drive the expression of Cas9 by an efficient caulimoviral promoter, 'M24' isolated and characterized from the *Mirabilis Mosaic Virus* (MMV). The 20-nucleotide CRISPR guide (g)-RNAs were designed to induce targeted mutations in the *CYP82E4-nicotine N-demethylase (nnd)* gene of tobacco (*Nicotiana tabacum*). For editing the *nnd* gene, we employed a pair of gRNAs followed by the protospacer adjacent motif (PAM) targeting the first exon of the *nnd*-ORF. We evaluated the percent "indels" using tobacco protoplast cells where mutagenesis frequencies were recorded as 45% and 30% for the two targets respectively. A mutagenesis efficiency of 37% was obtained upon the simultaneous transfection of the two gRNAs in tobacco protoplasts. Successful demonstration of our caulimoviral-based CRISPR-Cas9-gRNA system bodes well for its near-term use as a potential and facile means to performing targeted genome editing in plants.

Keywords: CRISPR; De-methylase; Protoplast; Tobacco

Introduction

The RNA-guided endonuclease activity of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated Cas9 nuclease in bacterial defence system has adapted along with the CRISPR guide RNA for biotechnology applications [1-5]. The other nuclease-based targeted gene disruption technologies including the zinc finger nuclease (ZFN) [6-8] and the transcription activator like effector nuclease (TALEN) [7,9] are also based on DNA-binding protein specificities. However, the ZFN and TALEN have certain difficulties associated with their designing and cost-effectiveness as compared to the CRISPR- Cas9 technology. Both ZFN and TALEN technologies are prone to some off-site DNA cleavage that may account for their reduced efficacies. Although, CRISPR faces some off-target risks, it is still less time consuming and has an ease of manipulation [10].

The Cas9 nuclease widely used for gene editing has been isolated from the type II bacterial CRISPR system of Streptococcus pyogenes having two independent nuclease domains and is capable of generating double-strand DNA breaks (DSB) on the specific site upstream of a PAM which is composed of a recognition sequence of two nucleotides (GG) [1-3,11,12]. The CRISPR-Cas9-gRNA system has been successfully tested in various cell types and organisms such as in bacteria [13,14], yeast [15], zebrafish [16-19], fruit fly [20], human cells [1,3,21] and both model and crop plants [22-26].

There have been tremendous advancements in the field of CRISPR-mediated gene-editing which has led researchers to devise some novel and efficient genetic tools to perform targeted gene modifications. The first step towards an effective editing strategy is the designing of suitable vehicles that can drive the expression of Cas9 along with the gRNAs. For this purpose, there are a series of vectors that can be used for plant transformation for introducing targeted mutations/insertions-deletions within the genome. Here, we report the designing and testing of a novel caulimoviral promoter driven CRISPR-based system for efficient gene editing.

Nicotine is the major alkaloid (90% of the total alkaloid pool) in all Nicotiana species [27-29]. Nornicotine, about 5% of the total pyridine alkaloid pool in tobacco, a N-demethylated derivative of nicotine, is accumulated in tobacco leaf during senescence and curing process of matured leaf. Nicotine is synthesized from aspartate and putrescine through several enzymatic steps, and then de-methylated by N-nicotine demethylase enzyme (*NND*) to nornicotine. There are reports of functional tobacco nicotine N-demethylase enzymes [30-33] which are members of the cytochrome P450 mono-oxygenase belonging to the CYP82E subfamily [31]. The major nicotine N-demethylase enzyme is CYP82E4. CYP82E4-*nnd* gene is developmentally regulated; its transcript level is high during senescence and air-curing of tobacco as well as under biotic stress [ethylene and tobacco mosaic virus (TMV) infection] treatments [34]. The two others functional *nnd* enzymes are CYP82E5v2 [35] and CYP82E10 [36]. Expression of CYP82E4 is leaf senescence specific whereas CYP82E5v2 and CYP82E10 are constitutively expressed in green leaves and root tissues respectively [35,36].

Based on the above facts, we sought to target the *nnd* CYP82E4 gene and thereby devise a system to induce targeted modifications within its exon sequence for its disruption. In previous studies, chemical mutagenesis, ethyl methane sulfonate (EMS) treated plants possessing mutations in nnd genes have shown reduced nicotine to nornicotine conversion [36]. The expression of RNAi constructs of CYP82E4 in tobacco plants also documented in reduction of nornicotine levels [37]. However, the genetic alteration of tobacco nnd

genes by the CRISPR-Cas9 system could be beneficial to minimize nornicotine formation.

In this study, we performed co-expression of Cas9 and two gRNAs for dysfunctioning the nnd gene in protoplasts from tobacco cell suspension culture. We designed binary vectors harboring Cas9 and a pair of gRNAs under the caulimoviral promoters M24 and FSgt3 respectively for dismantling the nnd gene. We specifically targeted the first exon of the nnd-ORF and evaluated the percent "indels" and calculated the mutagenesis frequencies on individual and simultaneous transfection of the two gRNAs in tobacco protoplasts. These newly adapted binary transformation vectors could become additional tools for targeted genome editing in planta.

Materials and Methods

Designing and synthesis of gRNAs

We designed chimeric gRNAs for targeting the nnd exon 1 (GenBank: KC120817.1) following the criteria described earlier [3,22]. The 'CCTop' CRISPR/Cas9 target online prediction tool was used for predicting the potential targets for nnd exon 1 [38]. We obtained several gRNA sequences for the target region from where we selected a pair of closely located representative gRNA sequences (20 bp). The off-target analysis was also performed for all the targets tolerating a maximum of two mismatches in the core sequence. The sequences of the two gRNAs (in lowercase) along with their PAM motifs (underlined) and scaffolds (in uppercase) used in this study are as follows:

gRNA111-134 (gRNA1):

5'CTGCAGcccggaggatggccggtaatcggGTTTTAGAGCTAGAAATAG CAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG GCACCGAGTCGGTGCTTTTTTTT-3' and gRNA190-212

(gRNA2):

5'CTGCAGgagacttagctgacaaatacggGTTTTAGAGCTAGAAATAG CAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG GCACCGAGTCGGTGCTTTTTTTT-3'.

Construction of genome-editing binary transformation vectors

The pSiM24 (GenBank: KF032933) vector which contains a strong constitutive promoter M24 [39] was used as the source generating material for designing the efficient binary transformation vectors used in this study.

The synthetic *Cas9* gene from *Streptococcus pyogenes* was obtained in pCAMBIA1302 from where it was PCR amplified using appropriate primer pairs

(FLAG-Cas9-FP:

5′-GCGGACCTCGAGccatggGTGATTACAAGGACGATGAT-3′,

NLS-Cas9-RP:

5′- ATGCAGgagctcTTACACCTTGCGCTTCTTC-3′).

The isolated *Cas9* gene was cloned into the *Xho*I and *Sst*I sites of the pSiM24 vector. The *Cas9* contained a 2X FLAG tag sequence (DYKDDDDKKDYKDDDDKH) at the 5'-end, for immuno-detection, and a SV40 nuclear localization signal (NLS; KKKRKV) at the 3'-end. The *Cas9* gene was flanked by the M24 promoter isolated from *Mirabilis mosaic virus* [40] and a 3'-terminator of ribulose bisphosphate carboxylase small subunit (rbcSE9). The designed vector was annotated as pSiM24-Cas9.

The designed gRNAs were synthesized (obtained in the pUC57 vector) and obtained as the pUC57-nndgRNA1 and pUC57-nndgRNA2 constructs. The gRNAs were then cloned in the pSiM24-Cas9 vector under the *Figwort mosaic virus* sub-genomic transcript (FSgt) promoter [41] and a CaMV 35S terminator (35S-T). The gRNA1 was inserted as a SmaI-FSgt3-nndgRNA1-35ST-*EcoR*I fragment into the corresponding sites of pSiM24-Cas9 to generate the plasmid pSiM24-Cas9-nndgRNA1. The gRNA2 was inserted as an *EcoR*I-FSgt3-nndgRNA2-35ST-*EcoR*I fragment into the *EcoR*I site of pSiM24-Cas9 to generate the plasmid pSiM24-Cas9-nndgRNA2. To clone both nndgRNA1 and 2 in a single binary vector, the *EcoR*I-FSgt3-nndgRNA2-35ST-*EcoR*I fragment was inserted into the *EcoR*I site of pSiM24-Cas9-nndgRNA1 to generate the plasmid pSiM24-Cas9-nndgRNA1-2.

Transient protoplast assays

The above constructs were introduced into healthy tobacco protoplasts (cv. Xanthi) using electroporation following a standard protocol [42]. Briefly, 10 µg of the respective plasmid DNA was electroporated in the protoplasts (~60,000 cells) and incubated for 36 hrs in dark. Each construct was transfected and assayed in at least three replicates.

Assays to evaluate the frequency of targeted modifications

To analyse the frequency of modifications induced by Cas9-nndgRNA, we designed primers that amplify a ~350 bp genomic region of the nnd exon 1 containing targets for both gRNA1 and 2 (nndexon1-FP: 5′-ATGCTTTCTCCCATAGAAGCC-3′, nndexon1-RP: 5′- AAGGTAATCGCCGTAAAGAAA-3′). The transfected protoplasts were transferred to microcentrifuge tubes and centrifuged gently at 100 g for 2 min. The supernatant for each sample was removed and the pellet was immediately frozen in liquid nitrogen. Next, the pellet was re-suspended in 40 µL of sterile water by intermittent vortexing. This was followed by heating the re-suspended protoplasts at 95°C for 10 min [43].

For analysis, 2 µL of the heated protoplast suspension was taken as the PCR template and the master mix was prepared in a total volume of 50 µL. The amplification of the target region was performed using Hi-Fidelity *Taq* Polymerase (Agilent Technologies, Catalog #600400) The target amplicons were then sequenced to detect the modifications. To visualize the modifications, the sequences were aligned with the native genomic target sequence and the percent indels (insertion/deletion) were calculated. The mutagenesis frequency (MF) was calculated using the formula:

MF=(Number of modified clones/Numbers of total clones sequenced) × 100%

Results and Discussion

The CRISPR/Cas9 system is a promising genome editing technology and is fast emerging as an alternative to classical plant breeding and transgenic (GMO) methods for improving crop plants. In a previous report, we proposed the use of CRISPR-Cas9-gRNA-mediated genome editing to dismantle the structure and expression of the nnd gene [44].

For this purpose, we created a few caulimoviral promoter-derived genome editing vectors that could express both Cas9 and gRNAs in the plant cells. Our strategy involved the assembling of both Cas9 and gRNA cassettes in a single binary expression vector. This strategy is most commonly used for generating efficient targeted mutations in the plant systems and has been reported in several instances [45,46]. In other studies, the CaMV35S promoter has been used to express the Cas9 gene and successfully target gene loci in plants [24]. The gRNA on the other hand is usually expressed under the DNA-dependent RNA polymerase III promoter (U6 promoter) while its expression is terminated by the U6 terminator.

In this study, we demonstrate the use of other members of the caulimoviral promoters to drive the expression of both Cas9 and gRNA. For this, we have employed the M24 promoter, which contains the duplicated enhancer domains from MMV to express Cas9 gene [39]. The M24 is a strong constitutive promoter, reported to have much higher activity than the CaMV35S promoter. The M24 promoter was used to design an efficient expression vector pSiM24 which has been tested and evaluated in both transient and transgenic plant platforms. The pSiM24 is a small, highly efficient and user-friendly binary gene expression vector which is widely being used for plant transformation with maximum efficacy [39,44]. Through this study, we wanted to check the implication of pSiM24 as a potential gene-editing vector. This will help to enrich the genetic tools that are available to perform genome editing using the CRISPR-Cas9-gRNA system.

The advantage of using pSiM24 is that it is useful for both transient and stable transgenic expression in plant systems. In various instances, the CRISPR-Cas9 system has been successfully used to modify the plant genome with high efficiencies [22-24,26].

However, there are a few constrains that are associated with the use of plant expression vectors, such as large size and low copy number. The optimal size and high copy number of the pSiM24-derived vector can help to overcome these drawbacks and improve the overall transfection efficiencies.

To evaluate the efficacy of our newly designed genome editing ensemble, we performed several assays using transient protoplast transfection and evaluated the modification efficiencies. For creating the Cas9-induced modifications, we first cloned the Cas9 gene and the gRNAs and created a series of vectors (Figure 1).

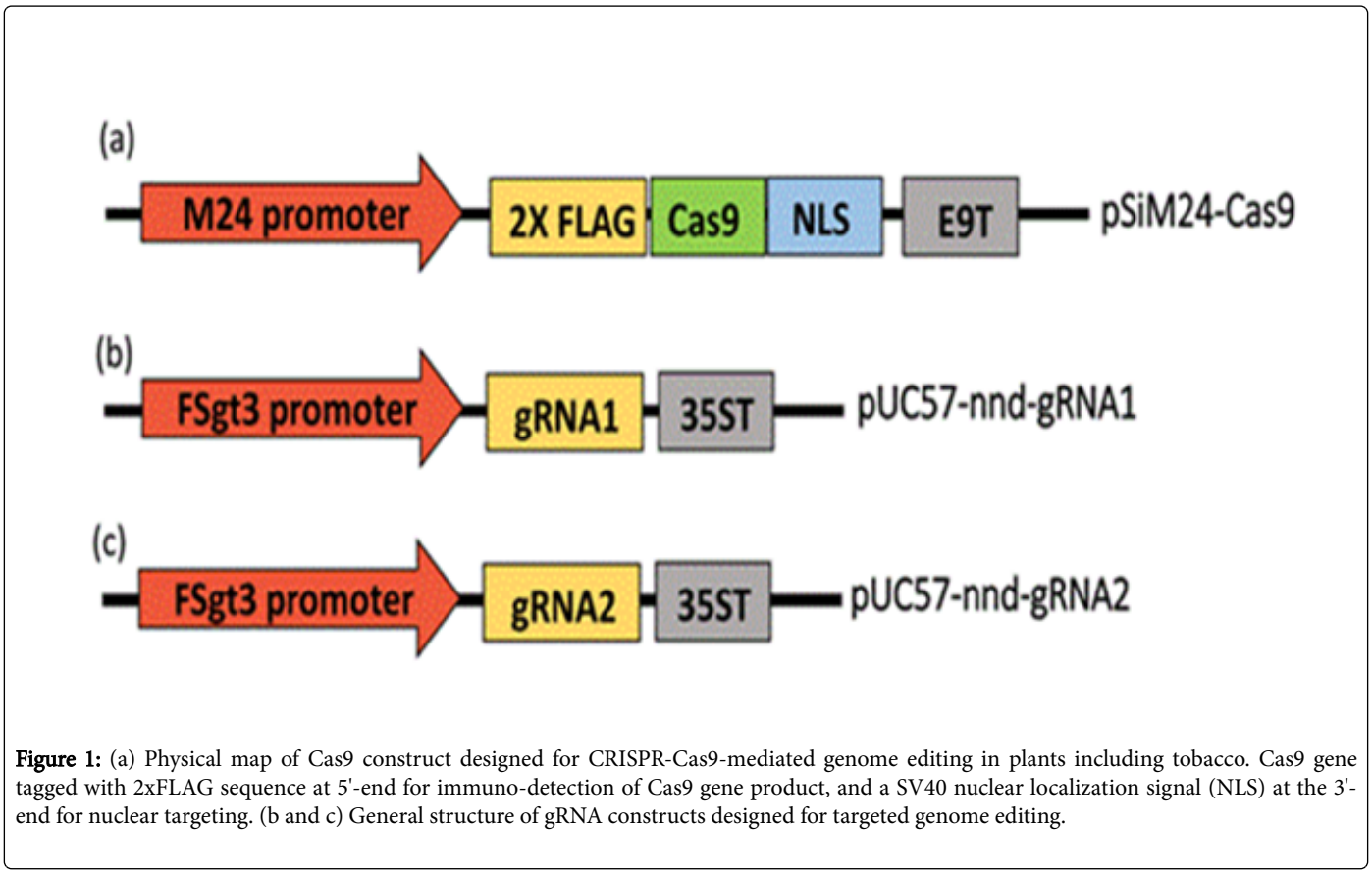

Figure 1: (a) Physical map of Cas9 construct designed for CRISPR-Cas9-mediated genome editing in plants including tobacco. Cas9 gene tagged with 2xFLAG sequence at 5'-end for immuno-detection of Cas9 gene product, and a SV40 nuclear localization signal (NLS) at the 3'-end for nuclear targeting. (b and c) General structure of gRNA constructs designed for targeted genome editing.

We designed suitable vectors harboring the Cas9 gene along with respective gRNAs in a single binary expression vector using the cloning strategies depicted in Figure 2. These vectors can be used for both transient as well as transgenic platforms to perform gene editing in planta.

To target the *nnd* gene, we first electroporated the construct pSiM24-Cas9-nndgRNA1 into healthy protoplasts and checked for the presence of any indels/mutations in the genomic DNA. The two target sequences with their respective PAM motifs are represented in Figure 3a. We sequenced a total of twenty amplicons out of which nine showed modifications in the target region.

Hence, the MF was calculated as 45% where all nine showed indels while three showed point mutations along with indels (Figure 3b).

Next, we transfected the tobacco protoplasts with pSiM24-Cas9-nndgRNA2 and evaluated the modification efficiencies. We sequenced twenty amplicons of which six carried indels in the amplified target

region and the MF was calculated as 30%. Out of the six modified sequences, all showed indels while three carried both point mutations and indels (Figure 3c).

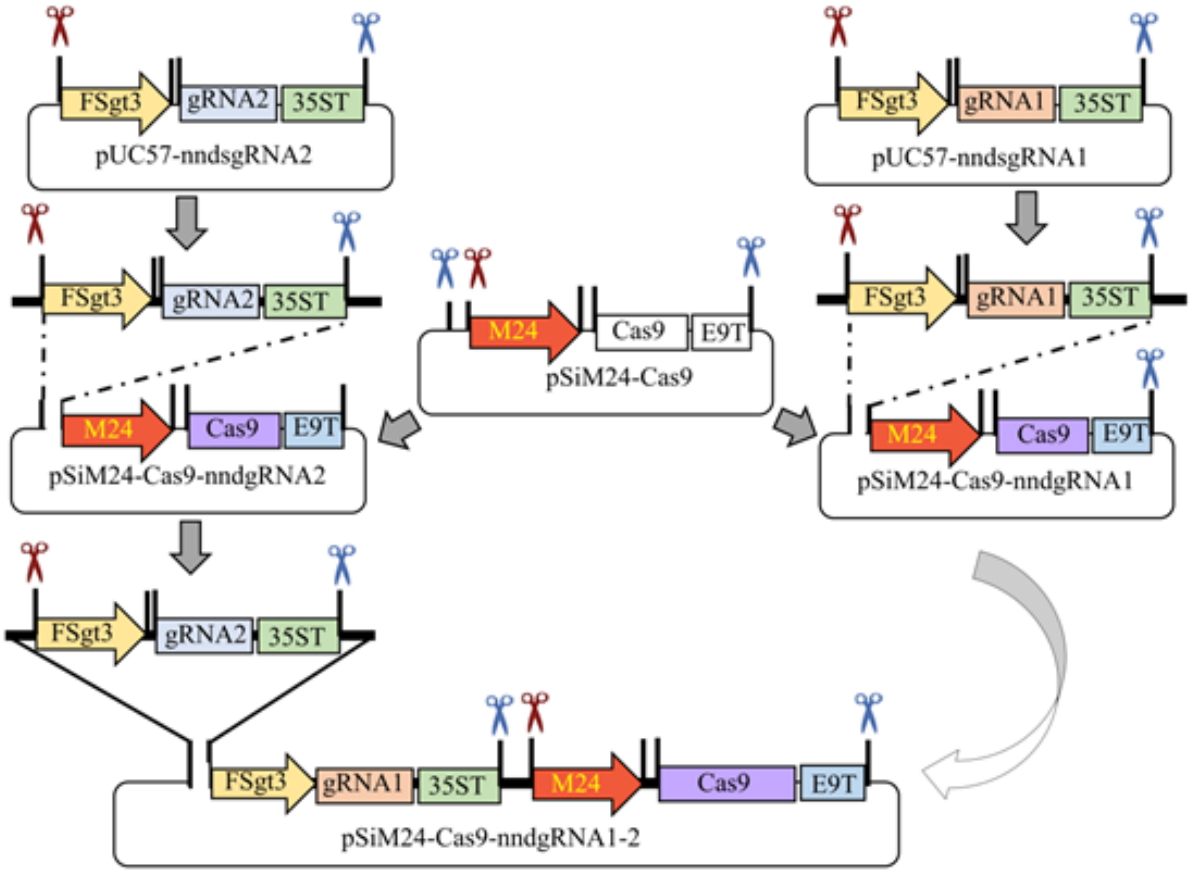

Figure 2: Schematic representation and cloning scheme for designing the test constructs. Physical map of three constructs designed for CRISPR-Cas9-mediated genome editing containing the Cas9 and gRNAs in a single binary vector.

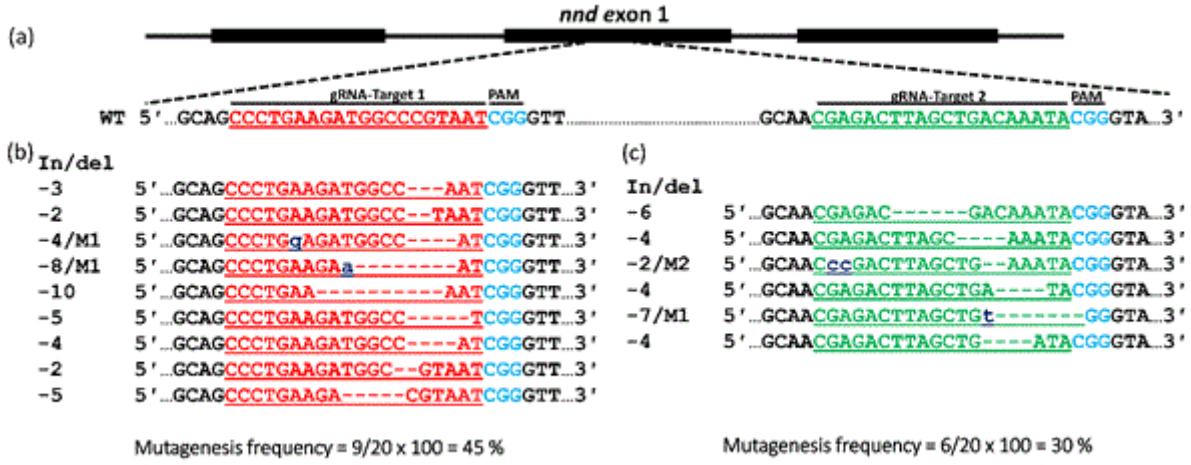

Figure 3: (a) *nnd* exon1 showing both target sequences where the 20-nt target is shown in underlined sequence and the PAM sequence is blue. (b) Targeted mutagenesis in tobacco protoplasts generated in target 1 of *nnd* exon1. (c) Targeted mutagenesis in tobacco protoplasts generated in target 2 of *nnd* exon1. Mutagenesis frequencies were evaluated separately for both targets and represented as MF.

To check whether the combined effect of both gRNAs could create a more profound effect, we electroporated the construct pSiM24-Cas9-nndgRNA1-2 into tobacco protoplasts and sequenced the target region. A total of eleven amplicons were sequenced out of which there were mutations in four and the MF was calculated as 37%. The indels were detected in both targets, where target 1 was more modified than target 2 (Figure 4). However, we did not detect any large fragment deletion that included both target regions.

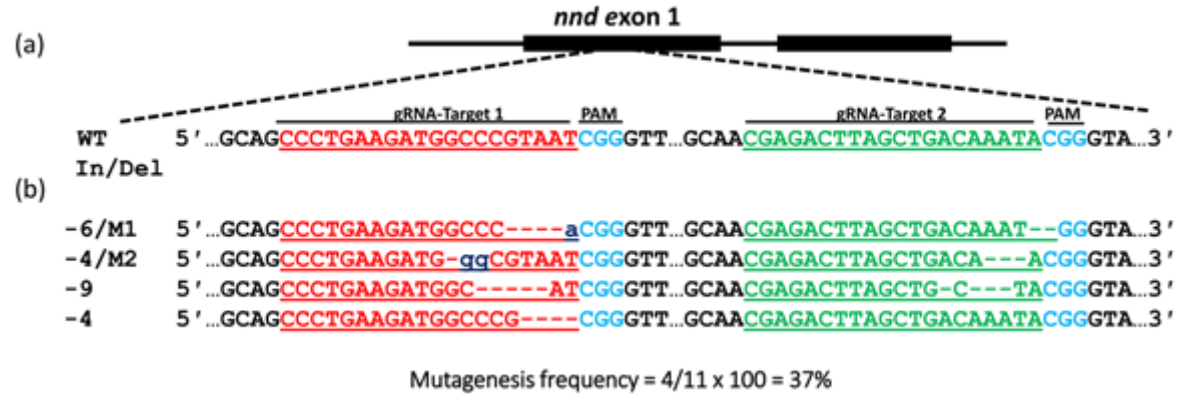

Figure 4: Combined targeted mutagenesis in tobacco protoplasts generated in target 1 and 2 of *nnd* exon1. Mutagenesis frequencies were evaluated separately for both targets and represented as MF after simultaneous transfection of both gRNAs.

The above results clearly demonstrate the high efficacy of the M24 promoter driven Cas9 in targeted genome modification. The positioning of gRNAs is a critical factor that decides the robustness of the system and how it can affect the overall efficacy of the editing system. Choosing closely spaced targets can inevitably increase the chances of getting a more pronounced effect on the overall gene disruption. The use of a pair of gRNAs in a single expression cassette may promote higher modification frequencies; however, it does not necessarily ensure a larger deletion in any segment. Our study holds promise for improving the genetic tools available for plant modification particularly for targeted gene editing. We speculate that while generating transgenic plants using our described vectors, the transformation efficiency can also be expected to be good with stable genetic modifications.

Conclusion

Our study focuses on adapting caulimoviral promoter-based vectors for CRISPR-Cas9-gRNA -mediated genome editing applications in plants. The work describes the development of molecular vectors harboring gRNA/Cas9 system for modification of tobacco nnd gene and evaluation of mutagenic activity of these vectors in protoplasts.

The caulimoviral promoters FSgt and M24 were used for gRNA and Cas9 genes respectively, where several types of mutations were registered in target sites.

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Summary Statement

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