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Design of single guide RNA for CRISPR/Cas9 mediated genome engineering in rice – for semi-dwarfism and thermo-sensitive genic male sterility

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Abstract
CRISPR/Cas9 causes high precision genome editing in an efficient manner. By designing an 18 to 20 nucleotide 'spacer' which is immediately followed by the protospacer adjacent motif, the Cas9 can be directed to cause double stranded breaks in a genome. The CRISPR plasmid pRGEB32 has been optimized to edit plant genomes. We designed sgRNAs to target the TMS5 and SD1 gene of rice using CRISPR-PLANT, an online platform for designing guide RNAs. The efficiency of the sgRNAs were assessed for various parameters such as GC content, potential off-target activity and specificity. The sgRNAs were cloned into the pRGEB32 plasmid background, transformed to competent E. coli and finally mobilized to AGL-1 strain of Agrobacterium. This protocol could be utilized to develop transformation ready CRISPR constructs in a short-time.

Keywords: CRISPR/Cas9, sgRNA, semi-dwarfism, thermosensitive genic male sterility

1. Introduction
CRISPR (clustered regularly interspaced palindromic repeats)/Cas (CRISPR associated) mediated genome engineering has rapidly advanced the crop improvement research mainly because of its relative simplicity and multiplexity than the other technologies such as ZFNs and TALENs (Ran *et al*. 2013) [12].

The CRISPR/Cas is an adaptive defence mechanism found in bacteria and archaea against the invading pathogens. The CRISPR/Cas works in three steps: incorporation of foreign genetic material into CRISPR locus (acquisition phase), transcription of these repeat sequences into pre-CRISPR RNAs (crRNAs; expression phase) and cleaving of invading plasmids or phage molecules by Cas nucleases by using the guide RNA sequences (interference phase). These double stranded breaks disrupt the invading DNA (Makarova *et al*. 2011) [10]. Based on the specific CRISPR-associated (Cas) proteins and non-coding RNA there are three major types (type I, II and III) and ten subclasses of CRISPRs were discovered (Carte *et al*. 2014) [1]. The type II system which involves Cas9 as the Cas-nuclease has been well utilized for genome editing.

The CRISPR/Cas9 system comprises of a non-coding transactivation CRISPR RNA (tracrRNA), an operon encoding the Cas proteins and a repeat array with a 20-nucleotide 5' targeting sequence and a 19-22 nucleotide repeat sequence known as 'spacers' (Deltcheva *et al*. 2011) [2]. In addition, the target sequence must contain a three nucleotide preceding sequence at the 3' end known as protospacer adjacent motif (PAM) (Jinek *et al*. 2012) [8] for the Cas9 protein to cleave. The commonly used CRISPR/Cas9 system of *Streptococcus pyogenes* requires the 5'-NGG-3' as the PAM (Zhang *et al*. 2014) [16].

The native CRISPR/Cas9 system has been optimized for genetic engineering. By designing spacers and combining with RNA complex (known as sgRNAs – single guide RNAs), the Cas9 can be directed to cause double strand breaks, given the target is immediately followed by the PAM (Jinek *et al*. 2012; Zhang *et al*. 2014) [8, 16]. The 'NGG' as PAM occurs ~1 in every 8 nucleotides making the target regions innumerable for a given genome. The induced double stranded breaks are repaired by the native repair mechanisms especially the error-prone non-homologous end joining (NHEJ) which often results in indels and frameshift.

To demonstrate, we have designed spacer targets for two loci in rice – *tms5* (*Os02g12290*) and

sd1 (Os01g66100) and cloned into the background of a CRISPR plasmid pRGEB32 (Xie et al. 2015) [15]. The various parameters that govern sgRNA design is compared and a successful mobilisation of the constructed plasmid into *Agrobacterium* strain AGL-1, amenable for transformation in rice.

2. Materials and methods

2.1 CRISPR/Cas9 cloning vector

The CRISPR/Cas9 vector plasmid pRGEB32 from Yinong Yang (Xie *et al*. 2015) [15] was acquired through Addgene (Addgene plasmid #63142; http://n2t.net/addgene:63142; RRID: Addgene_63142), USA by executing materials-transfer agreement and utilised in this experiment.

2.2 Design of sgRNAs

The oligos were designed using the online tool 'CRISPR – PLANT' targeting two gene regions namely 'tms5' (*Os02g12290*) and 'sd1' (*Os01g66100*) for development of thermosensitive genic male sterility and semi-dwarfism in rice varieties respectively. And the high purity salt free oligos were synthesized (Eurofins Genomics, India).

2.3 Primers for amplification of gRNA region of the plasmid

Primers targeting the gRNA region of the pRGEB32 was designed using 'Primer3-Web' (http://primer3.ut.ee/).

sgS_F: AGGCGTCTTCTACTGGTGCT

sgS_R: CCTCCGTATTTGCTGACGTG

2.4 Vector construction

The vector construction was performed as follows:

Enzymes used: 1. BsaI-HF enzyme (New England Biolabs, UK). 2. T4 DNA ligase (New England Biolabs, UK). 3. Dream Taq DNA polymerase (Themo Fisher Scientific, USA).

Linearization of the plasmid: The pRGEB32 plasmid was linearized using the BsaI enzyme. The following components were taken in a sterile 0.2 ml PCR tube and incubated in a thermal cycler (Applied Biosystems, USA) set at 37 °C.

Components	Volume (µl)
*Bsa*I-HF enzyme (1000 U)	1
Plasmid (250 ng µl^{-1})	3
Buffer	5
Molecular grade Water (Himedia, India)	41
Total	50
Incubated at 37 °C for 60 min	

The linearized plasmid was clarified in 1% agarose gel and gel purified using Favorprep gel/PCR purification kit (Favorgen, Taiwan). The concentration was adjusted to ~50 ng µl^{-1}.

Vector-oligo ligation: The linearized plasmid vector and the twin oligos were ligated as follows.

Components	*Volume (µl)*
Oligo_F (0.5 µM)	1.0
Oligo_R (0.5 µM)	1.0
T4 DNA ligase Buffer	2.0
Molecular grade water	13.0
The reaction mix was taken in 0.5 ml PCR tubes and incubated in 95 °C hot water for 4 min and incubated again in 70 °C water bath and left to cool under room temperature. Proceeded to ligation reaction.	
Linearized plasmid (50 ng)	2.0
T4 DNA ligase	1 µl
Total	20
Incubated at 37 °C for 2 hours.	

2.5 Confirmation of vector-oligo ligation

The vector-oligo ligation was confirmed through polymerase chain reaction with sgS_F and sgS_R primers followed by digestion with *Bsa*I enzyme.

2.6 Sanger sequencing

The ligation of the oligos in the vector background was confirmed using Sanger sequencing with sgS_F and sgS_R primers.

2.7 Transformation to competent *E. coli*

The DH5α strain of *E. coli* were made competent following the $CaCl_2$ method with minor modifications (Li *et al*. 2010) [9]. The plasmid was transformed into the *E. coli* by heat-shock for 45 seconds at 42 °C. The bacterium were grown with SOC medium for 1 hr. 100 µl of culture was spread on LB plates (with kinetin 50 µg ml^{-1}) and incubated at 37 °C for 16 hrs.

2.8 Colony PCR

Single colonies were picked and initially streaked on numbered fresh LB K50 plates with a pipette micro tip and washed in PCR tubes containing 25 µl of PCR master mix. After 30 cycles of PCR, the contents were clarified in 1% agarose gel.

2.9 Mobilisation into *Agrobacterium tumefaciens* for rice transformation

The super virulent strain AGL-1 was used in this study. The constructed CRISPR/Cas9 plasmid was mobilised in the *Agrobacterium* by triparental mating method (Ditta *et al*. 1980) [3] involving *E. coli* containing the CRISPR plasmid, *E. coli* containing pRK2013 (helper plasmid) and the AGL-1 strain. After incubating overnight, the bacteria were scraped and suspended in 0.9% NaCl. The cultures were serially diluted up to 10^{-6} and from each dilution 100 µl was plated on AB medium containing Rifampicin 20 µg ml^{-1} and Kanamycin 100 µg ml^{-1}. The successful mobilisation was further confirmed through colony PCR with sgS primers.

2.10 Glycerol stocks and plasmid storage

All the bacterial cultures *viz.*, pure strains of DH5α and AGL-1, DH5α with pRGEB32, DH5α transformed with the constructed plasmid containing sgRNA, DH5α with helper plasmid pRK2013, AGL-1 mobilised with the plasmid construct were stored as glycerol stocks (30% glycerol) and agar stabs with necessary antibiotics as long term and short term stocks with multiple replications. Glycerol stocks were stored at -80 °C and the agar stabs at 4 °C.

In addition, plasmids of pRGEB32, pRGEB32 with sgRNA construct and pRK2013 were extracted from the cultures in *E. coli* using the alkaline-lysis method and stored at -20 °C with multiple replications.

3. Results

The pRGEB32 CRISPR vector (Xie *et al*., 2015) [15] was used in this experiment (Fig 1). The plasmid construct has endogenous Cas9 with rice snoRNA U3 promoter to express the gRNA. Target specific spacers are designed and cloned into the vector background by digesting the plasmid with *Bsa*I enzyme (Fig 2a&b). The plasmid has Hygromycin as selection marker (in plants) and Kanamycin for bacterial resistance.

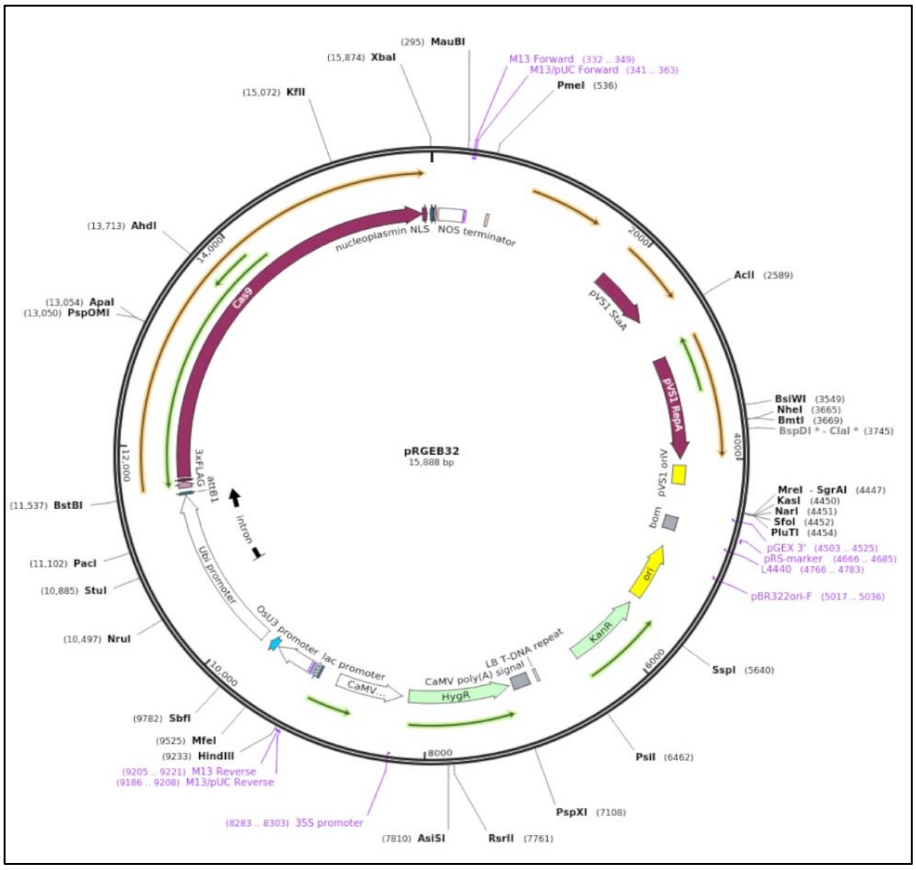

Fig 1: The plasmid map of the plasmid pRGEB32 optimized for plant CRISPR/Cas9 editing (Xie *et al*., 2015) [15]

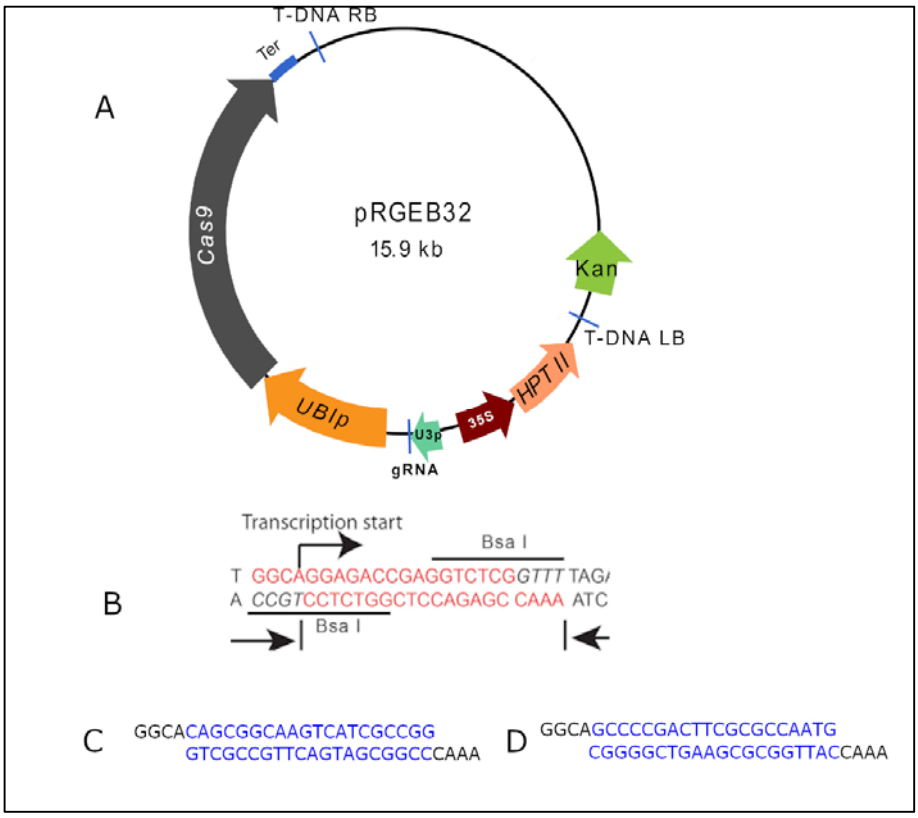

Fig 2: A. The pRGEB32 plasmid with endogenous *Cas9*, rice U3 promoter in the gRNA region, HPTII and Kanamycin selection markers; B. The *Bsa*I site in the gRNA where the spacers could be inserted; C & D. The oligos designed for the gene targets *tms5* and *sd1* (note: the 4 nt overhangs help in ligation with vector).

3.1 sgRNA design

The single guide RNAs targeting the thermosensitive genic male sterility and semi-dwarfism in rice were designed (Table 1).

Table 1: The sgRNA designed for targeting the rice genes

Target gene	Designed oligos	Sequence (5' to 3')	GC content (%)	Number of Gs & Cs in the last six sequences near PAM
tms5	tms5_F	GGCACAGCGGCAAGTCATCGCCGG	70.80	6
	tms5_R	AAACCCGGCGATGACTTGCCGCTG	62.50	5
sd1	sd1_F	GGCAGCCCCGACTTCGCGCCAATG	70.80	3
	sd1_R	AAACCATTGGCGCGAAGTCGGGGC	62.50	6

3.2 Vector-oligo ligation

The vector plasmid (linearized with *BsaI* enzyme) and the double stranded oligos were ligated together with the DNA ligase. The ligation reaction was confirmed through PCR (Fig 3c).

3.3 *E. coli* transformation

The homemade Dh5α competent cells were efficient in heat-shock method of transformation (Fig 3f). The observed single colonies were picked and confirmed with colony PCR, PCR/RE assay with *Bsa*I enzyme (Fig 3d) and Sanger sequencing.

3.4 Mobilisation into *Agrobacterium*

The constructed plasmid was mobilised into *Agrobacterium* by following the Triparental mating method (Ditta *et al*., 1980) [3]. The mobilisation was confirmed by growing the culture in double antibiotic media (Rifampicin 20 µg ml^{-1} + Kanamycin 100 µg ml^{-1}) and colony PCR (Fig 3A).

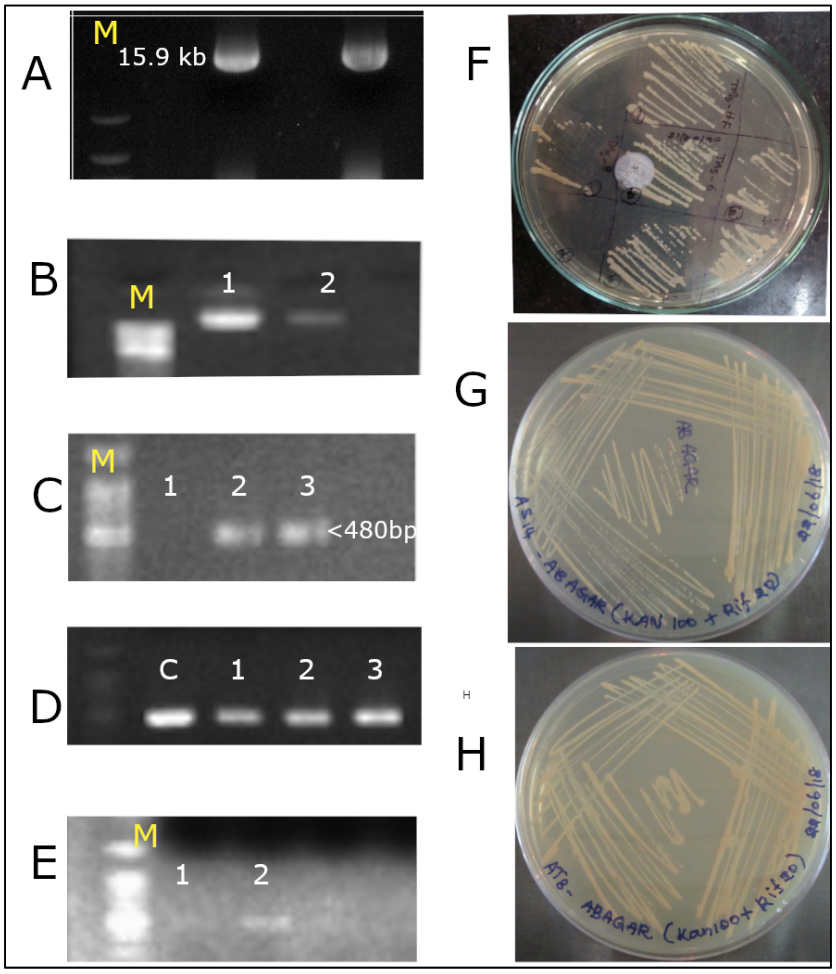

Fig 3: A. The CRISPR plasmid pRGEB32 (M – 100 bp ladder; the bands correspond to ~15.9 kb); B. BsaI digestion of pRGEB32 plasmid (M-ladder; 1-Uncut vector control; 2-digested and purified sample); C. PCR confirmation of the Vector-Oligo ligation (1-digested vector without insert (negative control); 2-sd1 construct; 3-tms5 construct); D. Colony PCR after successful *E. coli* transformation (C-positive control; 1,2 – sd1 constructs; 3-tms5); E. *Agrobacterium* colony PCR (1-sd1 construct (a faint band), 2-tms5 construct); F. Overnight incubation of single colony streaks of *E. coli* containing tms5 and sd1 constructs; G&H. *Agrobacterium* colonies (tms5 and sd1) growing on AB medium with double antibiotics.

4. Discussion

Strict guidelines are to be followed during design of sgRNAs to avoid any potential off-targets. The *S. pyogenes Cas9* can be used to target both the coding and non-coding strand of the DNA by targeting 5'-20nt-NGG-3' or 5'-CCN-20nt-3'. And to increase the chance of knock-out, the target region of the gene is preferably between 5-65% of the protein coding region (Doench 2017) [14]. Considering this fact, the third exon of the *sd1* region and first exon of the *tms5* was targeted. The pRGEB32, when digested with *Bsa*I leaves overhangs of 5'GGCANNN…3' and 5'…NNNCAAA3' (Fig 2b). These sticky ends improve ligation efficiency and ensure the right conformation of the oligos. Hence four nucleotides *viz.*, 5'GGCA…3' and 5'AAAC…3' were added at the 5' end of the forward and reverse oligos respectively (Fig 2c). Once these oligos are self-ligated, the sticky ends would efficiently bind with the linearized plasmid.

(Ren *et al*. 2014) [7] observed that the sgRNAs with at least four GCs in the six nucleotides closest to the PAM nearly always had more than 60% heritable mutation. Our designed oligos contain high GC content (Table 1) which could increase the chance of heritable mutation when transformed in rice.

To reduce the off-target binding, the number of mismatches should be kept minimum (less than 3 nucleotides) (Hsu *et al*. 2013; Ren *et al*. 2014) [7]. While designing the spacers, only the unique sequences from the output were chosen. Further, the nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the rice genome specifically had bound with the gene of interest. This suggests that the sgRNA were specific. However, off-target mutations even with a few nucleotide mismatches had been already reported (Fu *et al*. 2013) [5]. This needs to be further evaluated after the successful transformation in rice.

The proper and complete digestion of plasmid is necessary for successful ligation and to avoid false positives caused by uncut vectors. Digestion of the pRGEB32 plasmid with high-fidelity *Bsa*I enzyme for 1 hr ensured proper digestion (Fig 3b). The designed spacers (forward and reverse oligos) have sticky ends to enable easy ligation with the vector. The oligos were self-ligated by incubating in hot water and allowing to cool slowly to room temperature. The self-ligated oligos were then ligated to the vector background. The ligation reaction was confirmed through PCR (Fig 3c) with cut vector as negative control. Presence of bands in ligation templates and absence in cut-vector templates suggested successful ligation and complete digestion of vector plasmid respectively.

Triparental mating (Ditta *et al*. 1980) [3] is routinely used to mobilise the plasmid of interest from *E. coli* to *Agrobacterium* with the help of a helper plasmid pRK2013. The plasmid pRK2013 is self-transmissible and efficiently transfers between Gram-negative bacteria. It can promote the conjugal transfer of unrelated plasmids through triparental mating in two steps: transfer of the pRK2013 to the donor strain carrying the mobilisable plasmid followed by the expression of the genes necessary for conjugation in the donor and thus transfer of the mobilizable plasmid to the acceptor strain (Heinze *et al*. 2018) [6].

The AGL-1 is a hypervirulent *Agrobacterium* strain that transfers stable copies of expression plasmids into the plant cells (Wu *et al*. 2003) [14]. The plasmid construct (pRGEB32 + sgRNA) were successfully mobilised into the *Agrobacterium* by triparental mating. The appearance of multiple colonies in the double antibiotic selection media (Rifampicin 20 µg ml^{-1} + Kanamycin 100 µg ml^{-1}) confirmed the mobilisation. Further the colony PCR revealed the presence of the plasmids.

5. Conclusion

We report the design of single guide RNA for targeting rice *tms5* locus and *sd1* locus amenable for CRISPR mediated genome editing. The high GC content of the sgRNA, appropriate length (20 bp) and uniqueness to the target region ensures precise targeting and editing in the rice genome. However, probable off-target sites of mutations would be evaluated after transformation in rice. The sgRNAs were successfully cloned into the pRGEB32 plasmid with a single restriction enzyme *Bsa*I and successfully mobilised into the *Agrobacterium* strain. This protocol can be utilized to design and create a transformation ready CRISPR/Cas9 cultures in a short time.

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