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CRISPR/Cas9 Driven Targeted Editing of *Grain Number 1a* gene: sgRNA Constructs Design for Yield Enhancement in Rice (*Oryza sativa* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Yield is the ultimate trait determined by various quantitative trait loci (QTLs). One such QTL, *GRAIN NUMBER 1a* (*GN1a*), encodes for an enzyme Cytokinin oxidase/ dehydrogenase (CKX), which negatively influences the yield by degrading the phytohormone Cytokinin. CRISPR/Cas9, a precise means for targeted editing of a gene for the improvement of a particular trait in plants. Therefore, targeted genome editing of *GN1a* gene was performed to down-regulate the expression using

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CRISPR/Cas9 for the increase in grain number and yield with improved Cytokinin content in the panicle meristem. For the site-targeted mutagenesis, the single guide RNAs (sgRNAs) were designed using plant specific CRISPR-P v2.0 software. Two efficient sgRNAs were chosen critically entrenched on their GC content, on-target values, location on the gene, off-target sites and their location, secondary structures, adjacent to the Protospacer Adjacent Motif (PAM) NGG. The binary vector pRGEB32, with Cas9 influenced by rice ubiquitin promoter and *Bsa*I restriction site driven by rice U3 promoter was employed for cloning of sgRNAs. The sgRNAs were tempered, phosphorylated and astringed with the binary vector pRGEB32, transformed into *E. coli* DH5α initially, then mobilized into *A. tumefaciens* EHA105. The present study helps in the development of elite lines which will lead to enhancement of grain number and overall yield for the growing population and farmer's welfare.

Keywords: Yield; Cytokinin; GN1a gene; genome editing; CRISPR/Cas9.

1. INTRODUCTION

Rice *(Oryza sativa* L.*)* is considered to be one of the most significant and primary cereal food crop, which provide sustenance for almost half of the world's population. Due to the continuous population growth in Asia, Africa, and Latin America, there will be a significant increase in the demand for rice. It is predicted that in order to meet the food demands of a growing global population, rice production will need to enhance proportionately (Wang et al.*,* 2022). New superior varieties with unique plant structures which can produce more grain yields are required to meet this growing demand. An example of this can be seen in the "green revolution," where lodging-resistant semi-dwarf varieties of cereals like wheat and rice have significantly increased grain yields (Peng et al.*,* 1999; Shah et al.*,* 2019; Wei et al., 2022).

Yield is the most complex and important physiological trait governed by various external (abiotic, biotic) and internal factors (genetic, biochemical, etc.,). The yield of rice in the year 2023-2024 is 4.2 T/ha which was decreased from the year 2022-2023 which was 4.3 T/ha (https://ipad.fas.usda.gov/updated on April 11, 2024). Reduction in the yield can be caused by physiological, environmental, and morphological bottlenecks which have huge impact on the growth and development of plant.

Cytokinins (CK) have a unique role in the growth and development of various organs. Plants developed precisely control in their concentration by taking benefit of biodegradation at spatial and temporal levels. 11 *CKX* genes have been distinguished so far in rice; but, the majority of their tissue specific activities persist unknown (Ashikari et al.*,* 2005; Zalabak et al.*,* 2016; Chen et al.*,* 2020), and were grouped together as clades. The first clade is comprised of *OsCKX1* and *OsCKX2*. *OsCKX1* is expressed in the apex of axillary buds as well as at the base of the shoot. Conversely, *OsCKX2*, typically found at elevated degree in the leaf collar and flowering, was also observed to be significantly expressed in the lateral root anlage, shoot plinth, and leaf blade. The second clade consists of *OsCKX6*, *OsCKX7*, and *OsCKX10,* which were exhibited very low expression level across every single tissue (Rong et al.*,* 2022).

The third clade included *OsCKX4*, *OsCKX5*, and *OsCKX9*. *OsCKX4* is usually highly expressed in vegetative tissues but expression specifically seen in the roots (Gao et al.*,* 2014; Rong et al.*,* 2022). The *OsCKX9* was normally expressed at a low concentration in almost all tissues but unveiled heightened expression throughout axillary buds and leaf blade (Duan et al.*,* 2019).*OsCKX5* is typically shown high expression across all the organs, but it showed specific expression patterns in the leaves and roots. The *OsCKX3* and *OsCKX8* were included in the fourth clade. Although *OsCKX3* was anticipated to be most highly expressed in the shoot and young panicles, it also exhibited significant expression in base of the shoot. The *OsCKX8*, a gene tends to have lower articulation levels in all vegetative organs than *OsCKX3*, displayed distinctly higher expression near the shoot outgrowth, in the primordia of flag leaf, and efflorescence. The *OsCKX11* showed a distinct nucleotide sequence compared to the other *OsCKX* genes included in the separate i.e., fifth clade and was generally expressed at greater levels in almost every tissue, especially in the roots, base of the shoot, and early flowering (Zhang et al.*,* 2021a; Rong et al.*,* 2022).

Cytokinins play a prominent role in the ordinance of panicle architecture which determines grain

number in rice (Azizi et al., 2015; Yeh et al., 2015). A QTL (Quantitative Trait Locus) that governs the grain yield in rice is *GRAIN NUMBER 1a* (*GN1a*) (Ashikari et al., 2005). Later it was found as a gene that encodes an enzyme Cytokinin oxidase/dehydrogenase (CKX), which degrades plant hormone Cytokinin and maintain its homeostasis (Ashikari et al., 2005).*CKX2/ GN1a* was the first CKX gene to be known. Decreasing the expression of *OsCKX2* or *Osckx2* mutations resulted in plants or varieties that produced more vegetative tillers, grain number per inflorescence, and heavier grains (Ashikari et al., 2005; Yeh et al., 2015; Li et al., 2016). The disruption of *OsCKX2* function increased the grain number, promoted secondary panicle branches, and boosted total grain yield by enhancing Cytokinin levels in the panicle tissue (Rashid et al., 2024).

In drought conditions, the *Osckx2* mutant retained more water and exhibited better water-saving characteristics, along with a survival response to manage dehydration stress. Additionally, *Osckx2* preserved the integrity of chloroplast membranes and demonstrated a notable improvement in photosynthetic function with enhanced antioxidant protection mechanisms (Rashid et al., 2024). The elevated expression of *OsCKX2* adversely affects the spikelet count per panicle and drought resistance, but does not have a noticeable effect on salinity tolerance. These reports suggest the potential of *Osckx2* mutant to develop climate-resilient high-yielding varieties (Rashid et al., 2024).

Now-a-days genetic engineering techniques such as gene silencing methods (Anti-sense technology, RNAi technology), and gene knock-out techniques (ZFN's, TALEN's, CRISPR/Cas system) are being used as efficient and precise tools for the development of elite varieties. Among these, CRISPR/Cas system is most widely used because of its high target specificity, and silencing efficiency.

Jansen et al. (2002) defined CRISPR as tandem repeats, adjoined with non-recurrence DNA segments that were initially identified as defense mechanism of prokaryotes for bacteriophages (Ishino et al., 1987; Horvath and Barrangou, 2010; Tahir et al., 2020). The components of CRISPR technology for the cleavage process are (i) a sgRNA, a synthetic oligo of 20 base pairs that align to the desired DNA and (ii) a Cas9 nuclease enzyme that cut three bases before the PAM (generally 5′ NGG; Jinek et al., 2012). It comprises of two domains, (a) RuvC-like domain (member of RNase H family) and (b) an HNH domain (member of HNH endonuclease family), each cutting one DNA strand (Nussenzweig and Marraffini, 2020).

2. MATERIALS AND METHODS

2.1 CRISPR/Cas9 System Binary Vector

The pRGEB32, binary vector of CRISPR/Cas9 system was procured from Addgene, the non-profit plasmid repository and obtained as Bacterial stab culture. The vector encapsulated in the culture was retrieved on the Luria-Bertani (LB) agar with antibiotic Kanamycin (50 mg/L).

2.2 Bacterial Strains

The *E. coli*, *A. tumefaciens* strains DH5α and EHA105 respectively were used in the study. The cultures were streaked and restored on LB agar plates with Nalidixic acid (25 mg/L) and Rifampicin (25 mg/L) antibiotics respectively.

2.3 Retrieval of Gene Sequence Data of *GRAIN NUMBER 1a* Gene

Rice *GRAIN NUMBER 1a* gene sequence was downloaded from Rice Genome Annotation Project (http://rice.uga.edu/) and Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/) in FASTA format and saved for further analysis. The Locus ID of the genome sequence for possible protospacer targets was taken from Rice Annotation Project Database.

2.4 Designing of sgRNAs

The sgRNAs were designed using plant specific CRISPR-P v2.0 software (http://crispr.hzau.edu.cn/). Two sgRNAs were chosen dependent on the off-target sites and their location, on-target scores, GC content, location on the gene, secondary structure (https://rna.urmc.rochester.edu/RNAstructure.html) of sgRNAs with the Protospacer Adjacent Motif (PAM) as NGG. The sgRNAs were synthesized as sense and antisense strands with suitable complementary restriction enzyme (*Bsa*I) sites as mentioned in Table 1.

2.5 Validation of sgRNAs

The insilico validation was done for the selected sgRNAs by secondary structure prediction using

RNA secondary structure prediction tool by Mathews Lab (https://rna.urmc.rochester.edu/).

2.6 Synthesis of sgRNAs

The sgRNAs were analyzed and synthesized by Integrated DNA Technologies (IDT) with *Bsa*I restriction sites.

2.7 Designing of Primers for the Study

Primers for M13 sequence of pRGEB32 vector and Hygromycin resistant gene (*hpt*) were designed manually using the assistance of OligoAnalyzer" tool of IDT as shown in Table 6.

2.8 CRISPR/Cas9 Binary Vector Construct

In view of construction of CRISPR/Cas9 genome targeted editing cassette in binary vector, the pRGEB32 vector was isolated with GeNei® Puresol™ plasmid isolation Kit and was linearized with restriction enzyme *Bsa*I-HF-v2, procured from New England Biolabs (NEB), UK. The restriction digestion reaction set up followed as mentioned in Table 2. The restriction digested pRGEB32 vector was examined on 1% agarose gel for linearization and then vector was cleaned up with PCR Purification Kit purchased from QIAGEN QIAquick® PCR Purification Kit. The quality and quantity was analyzed using Nanodrop® (IMPLEN NP80) Spectrophotometer.

2.9 Ligation

The complementary strands of sgRNAs (100µM each) were annealed and the 5' ends were phosphorylated using T_4 Polynucleotide Kinase (PNK) as mentioned in Table 3. The annealed phosphorylated sgRNAs were diluted in (1:200) ratio and were ligated into digested pRGEB32 vector and then cloned into *E. coli* DH5α competent cells as mentioned in Tables 4 and 5.

Table 1. The sgRNAs for targeted gene editing of *GN1a* with *Bsa*I sites

Sl. No	Gene and sgRNA	Strand	Sequence (5' - 3')	PAM
1	*GN1a* sgRNA 1 (OsGN1a # G1)	Sense strand Antisense strand	GGCAGCACGACGCGCGCAGCAGCG AAACCGCTGCTGCGCGCGTCGTGC	CGG
2	*GN1a* sgRNA 2 (OsGN1a # G2)	Sense strand Antisense strand	GGCACGAGTGGCCACACCCCCGCG AAACCGCGGGGGTGTGGCCACTCG	CGG

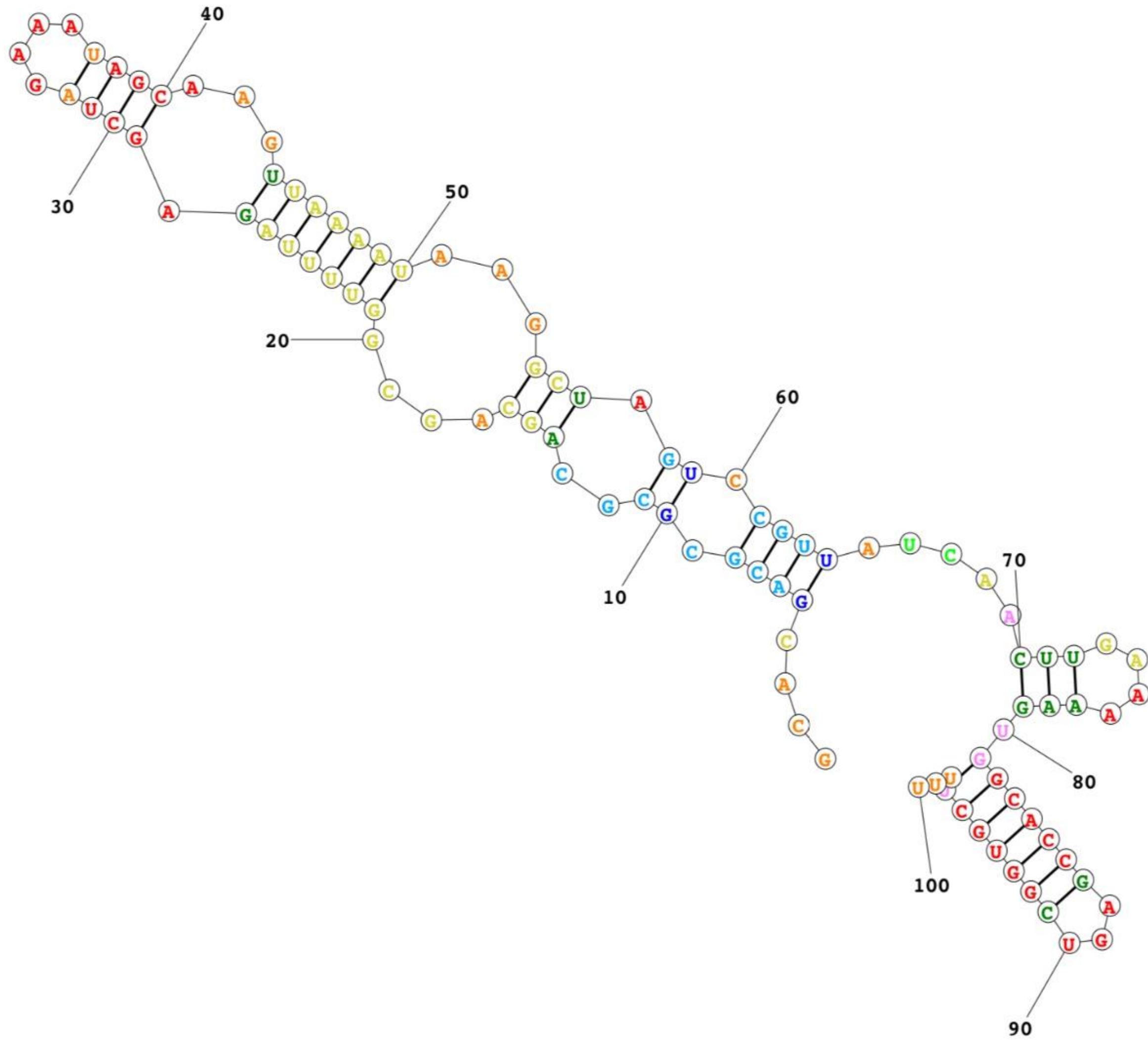
Table 2. Restriction digestion reaction set up of pRGEB32 vector

Components	Volume (µL)	Reaction conditions
Autoclaved distilled water	*to make up to 50 µL	Incubate at 37°C for 1 hr
10X CutSmart buffer	5	Heat inactivation at 65°C for 10min
*Bsa*I-HF-v2 enzyme	1	
pRGEB32 vector	*5 micro gram	
Total reaction volume	50	

Table 3. Annealing and phosphorylation of sgRNAs

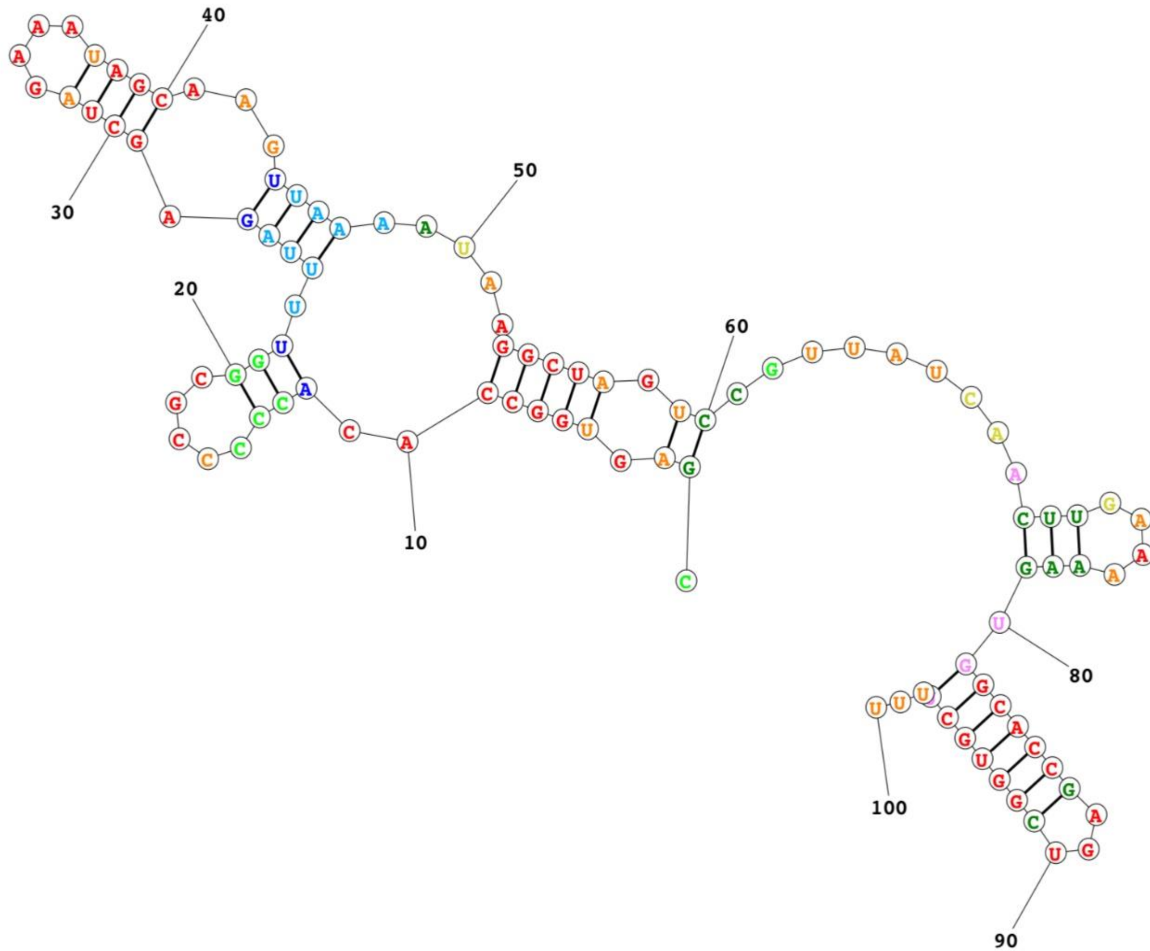
Components	Volume (µL)	Reaction conditions
Autoclaved distilled water	6.5	Incubation at 37°C for 3 min
10X PNK Buffer	1.0	Incubation at 95°C for 5 min, slowly cooling down to 25°C at Ramp rate of 0.1°C/sec
sgRNA oligo sense strand (100µM)	1.0	
sgRNA oligo antisense strand (100µM)	1.0	
T_4Polynucleotide Kinase enzyme (PNK)	0.5	Store at -20°C
Total reaction volume	10	

A.



Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability

B.



Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability

Fig. 1. A. Predicted secondary structure of sgRNA1 (OsGN1a#G1); B. Predicted secondary structure of sgRNA2 (OsGN1a#G2)

Table 4. Dilution of sgRNAs

Components	Volume (µL)	Reaction conditions
Annealed phosphorylated sgRNA	1	Mix it thoroughly
Autoclaved distilled water	199	Store at 4°C
Total reaction volume	200	

Table 5. Ligation of annealed sgRNAs and linearized vector

Components	Volume (µL)	Reaction conditions
Autoclaved distilled water	0.0	Incubation at 25°C for 15 min
10X ligation buffer	1.0	Store at 4°C for shorter time
Linearized vector (50ng)	7.0	Transformation into *E. coli* (DH5α strain)
T_4 DNA Ligase	1.0	competent cells
Diluted annealed PO_4^- sgRNA	1.0	
Total reaction volume	10	

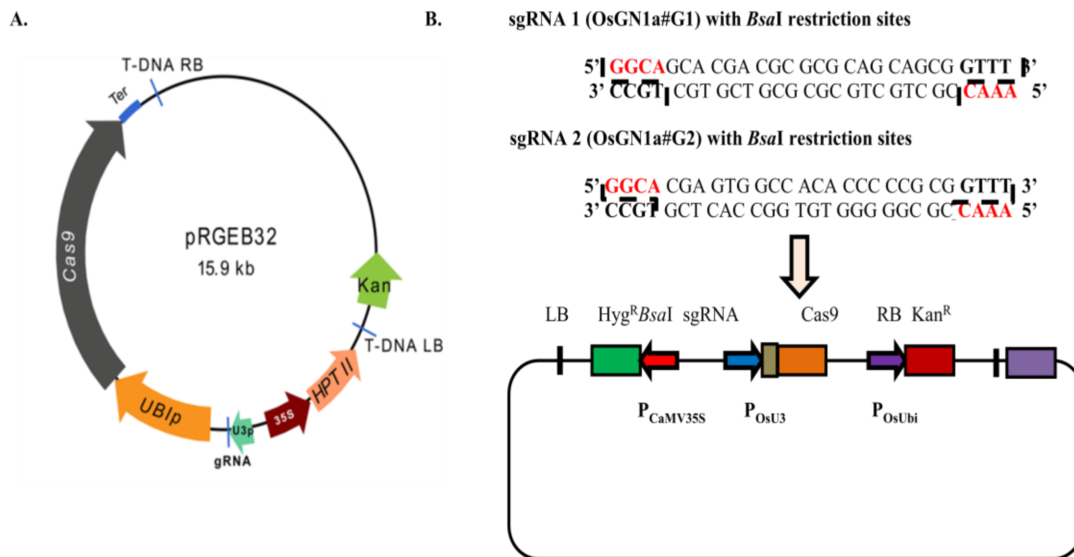


Fig. 2. A. Schematic representation of pRGEB32 vector; B. Cloning of sgRNAs between *BsaI* restriction sites expressed under OsU3 promoter

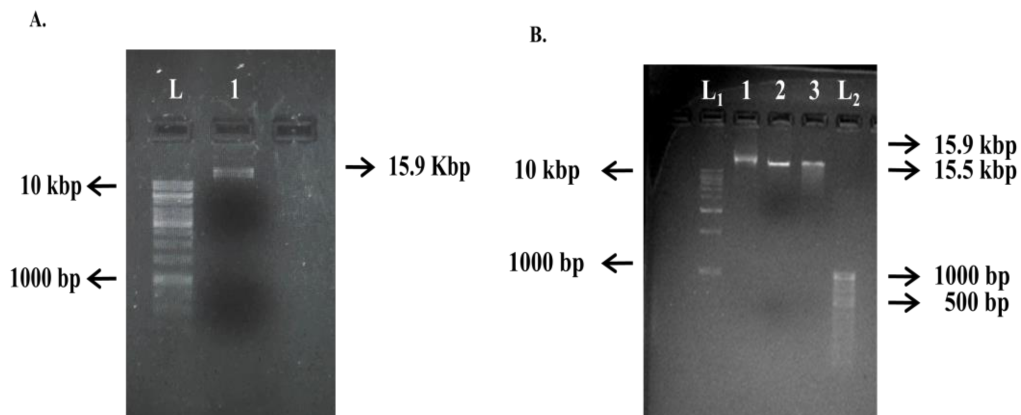


Fig.3. A. Gel picture of isolated pRGEB32 vector. L - 1kb ladder, 1 - pRGEB32 vector. B. Gel picture of *BsaI* restriction site profile of pRGEB32 vector. L_1 - 1kb ladder, 1 - undigested vector, 2 and 3 - digested vector, L_2 - 100bp ladder

2.10 Transformation of *E. coli* with Recombinant Vector

The ligated product consists of pRGEB32 vector incorporated with sgRNA was transformed into *E. coli* (DH5α strain). For this process, the competent cells were prepared by Calcium-Chloride ($CaCl_2$) method (Tang et al., 1994; Chang et al., 2017) and transformation was done through heat-shock method @ 42° C for 90 sec (Chang et al., 2017). After transformation, the bacterial culture was plated on LB agar containing selection marker Kanamycin (50 mg/L), with 'L' shaped spreader and incubated at 37° C for 16 h.

2.11 Confirmation of Recombinant Construct in *E. coli*

The positive clones were randomly selected, streaked onto fresh LB agar plates containing Nalidixic acid (25 mg/L) and Kanamycin (50 mg/L) and analyzed by colony PCR. The PCR was performed through KAPA *Taq* PCR kit (Merck, USA) by using M13 reverse primer as forward primer and sgRNA antisense oligo as reverse primer as mentioned in Table 7 and 8 and the PCR product was visualized on 1% agarose gel using gel documentation system. Bands of expected size ~450 bp were observed. The positive clones confirmed by colony PCR was used for plasmid isolation.

Table 6. Primers used for the research

Sl. No.	Purpose	Primer	Sequence (5'-3')
1	For PCR of recombinant constructs and sequencing plasmid clones	M13_R	CACACAGGAAACAGCTATGACCATG
2	Primers for amplifying Hygromycin resistant gene (*hpt*) (Partial)	Hyg_F	ACGTCTGTCGAGAAGTTTCTGATCG
		Hyg_R	CCGTCAGGACATTGTTGGAGC

Table 7. Colony PCR Master Mix

Components	Volume (µL)
Autoclaved distilled water	14.25
10X buffer A (Forward reaction)	2.0
25 mM $MgCl_2$	0.5
10 mM dNTP's	1.0
5U/µLTaq	0.25
10 µM primer F (M13 R)	1.0
10 µM primer R (sgRNAs)	1.0
Total reaction volume	20
Touch the colony edge with pipette tip or sterile tooth pick Tap in the reaction mixture	

Table 8. Colony PCR program

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	10 min	
Denaturation	95	30 s	38 cycles
Annealing	60 – 62	30 s	38 cycles
Extension	72	20 s	38 cycles
Final Extension	72	10 min	
Final Hold	4	∞	

Table 9. Plasmid PCR master mix

Components	Volume (µL)
Autoclaved distilled water	12.05
10X buffer A	2.2
25 mM $MgCl_2$	0.5

Components	Volume (µL)
10 mM dNTP's	1.0
100% DMSO	2.0
5U/µLTaq	0.25
10 µM primer F (M13 R)	1.0
10 µM primer R (sgRNA)	1.0
Recombinant vector	2.0
Total reaction volume	22

Table 10. Plasmid PCR program

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	10 min	
Denaturation	95	30 s	38 cycles
Annealing	60 and 62 (gradient)	30 s	
Extension	72	1.5 min	
Final Extension	72	10 min	
Final Hold	4	∞	

2.12 Re-confirmation of Recombinant Construct in *E. coli*

The plasmid was isolated by Alkaline-lysis method (Ehrt and Schnappinger, 2003) and re-confirmation of Recombinant construct was done by plasmid PCR. The quantity was analyzed using Nanodrop® Spectrophotometer, performed gel electrophoresis using 1% agarose and visualized under gel documentation system. The recombinant plasmid was also confirmed by PCR with KAPA *Taq* PCR kit by using M13 reverse as forward primer and sgRNA antisense oligo as reverse primer as mentioned in Table 9 and 10, and the product of PCR was visualized on 1% agarose gel using gel documentation system. Expected band size ~450 bp were seen. Further, the recombinant vector was confirmed by Sanger sequencing by GeneSpec Pvt. Ltd (https://www.genespec.com). The recombinant vector isolated was further purified using PCR purification kit purchased from QIAGEN QIAquick® PCR purification kit.

2.13 Mobilization of Positive Clones to *A. tumefaciens* Strain EHA105

A. tumefaciens strain EHA105 culture was prepared to be competent by $CaCl_2$ method. The confirmed recombinant vector cloned and isolated from *E. coli* was then introduced into EHA105 strain following freeze-thaw method @ 37°C for 5 min (Holsters et al., 1978). After transformation, the bacterial culture was spreaded on LB agar plates containing the antibiotics Rifampicin (25 mg/L) and Kanamycin (50 mg/L).

2.14 Confirmation of Recombinant Vector in *A. tumefaciens* Strain EHA105

The positive clones were randomly selected, streaked onto fresh LB agar plates containing Rifampicin (25 mg/L) and Kanamycin (50 mg/L) and analyzed by colony PCR. The PCR was performed through KAPA *Taq* PCR kit by using M13 reverse as forward primer and sgRNA antisense oligo as reverse primer; *hpt* primers and the product of PCR was visualized on 1% agarose gel using gel documentation system. The expected band size ~450 bp and ~600 bp were observed respectively. The positive clones were confirmed by colony PCR was used for plasmid isolation.

The protocols mentioned above were followed for colony PCR, plasmid isolation and plasmid PCR.

3. RESULTS AND DISCUSSION

3.1 CRISPR/Cas9 System Binary Vector

The size of CRISPR/Cas9 system binary vectorpRGEB32 is 15.9kbp (Xie et al., 2014) and consisted of *Bsa*I enzyme restriction site for sgRNA cloning, bacterial and plant selection markers Kanamycin (Kan^R) and Hygromycin (Hyg^R) resistant genes respectively under CaMV35 promoter, presence of Cas9 gene driven by rice ubiquitin promoter and also sgRNA cloned under pol III type promoter of rice U3 snoRNA as shown in Fig 2A.

3.2 Retrieval of Gene Sequence data of *GRAIN NUMBER 1a* gene

The locus ID for *GN1a* gene sequence was designated in RGAP database as

LOC_Os01g10110 and the gene was identified on chromosome 1 (Reverse orientation) (http://rice.uga.edu/). The complete sequence of the gene was downloaded in FASTA format from The Rice Annotation Project Database (RAP-DB) (https://rapdb.dna.affrc.go.jp/). The gene sequence length was 5576 bp, with a coding sequence of 1698 bp, covering 565 amino acids. The potential function was given as Cytokinin degradation. The gene had four exons and three introns (http://rice.uga.edu/).

3.3 Designing of sgRNAs

The sgRNAs of 20bp length for the specific targeting of *GN1a* were chosen from CRISPR-P v2.0 mentioned in Table 1. The two sgRNAs grounded on their on-target scores, GC content, location and position on the gene, off-target sites and their location, secondary structure were selected. The sgRNAs were located on the coding sequence region (CDS region) of the gene. The restriction sites of the type II restriction enzyme *Bsa*I were added to the 5'end of both sense and antisense strand of sgRNA to empower the cloning into pRGEB32 vector as shown in Fig 2B.

3.4 Validation of sgRNAs

The secondary structure showed the percentage probability of complementarity within the sgRNA and also the free 5' end for efficient adhering to the targeted genomic DNA as shown in Fig 1A and B that may knock-out the gene proficiently, thereby increasing grain number and overall yield.

3.5 Confirmation of Recombinant Construct in *E. coli*

The isolated plasmid and restriction digested plasmid with *Bsa*I was analyzed for linearization on 1% agarose gel as shown in Fig 3A and B. The restriction digested product was later ligated with annealed, phosphorylated sgRNAs; and were used for transformation into *E. coli* DH5α. After overnight incubation, bacterial colonies with constructs were observed on the LB agar plates containing Nalidixic acid (25 mg/L), and selection marker Kanamycin (50 mg/L). The positive clones were randomly selected, streaked onto fresh LB agar plates containing Nalidixic acid (25 mg/L) and Kanamycin (50 mg/L) and analyzed by colony PCR. Expected bands of size ~450 bp were obtained on 1% agarose gel as shown in Fig 4 and 5A. The positive colonies from PCR were chosen, used for plasmid isolation, plasmid PCR (Fig 4 and 5B) and Sanger sequencing performed using universal M13 reverse primer and the Sanger sequencing result data was analyzed and sgRNAs insertion was confirmed using sequence alignment editor BioEdit7.2 software. Colony number 4 of sgRNA1 (pRGEB32:OsGN1a#G1) and colony 1 of sgRNA2 (pRGEB32:OsGN1a#G2) displayed insertion of respective sgRNAs with in pRGEB32 vector as shown in Fig 6A and B.

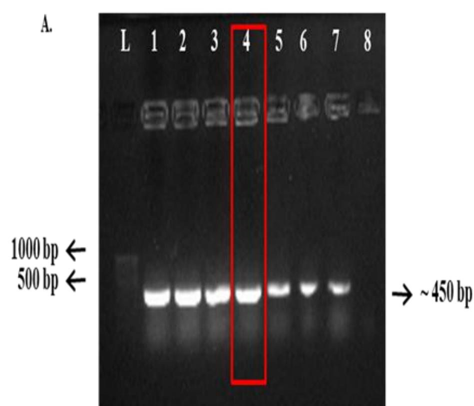


Fig. 4. A. Colony PCR outline of *E. coli* DH5α strain with the insertion of sgRNA

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G1-1, 2 - pRGEB32:OsGN1a # G1-2, 3 - pRGEB32:OsGN1a # G1-3, 4 - pRGEB32:OsGN1a # G1-4, 5 - pRGEB32:OsGN1a # G1-5, 6 - pRGEB32:OsGN1a # G1-6, 7 - pRGEB32:OsGN1a # G1-7, 8 - pRGEB32:OsGN1a # G1-8.

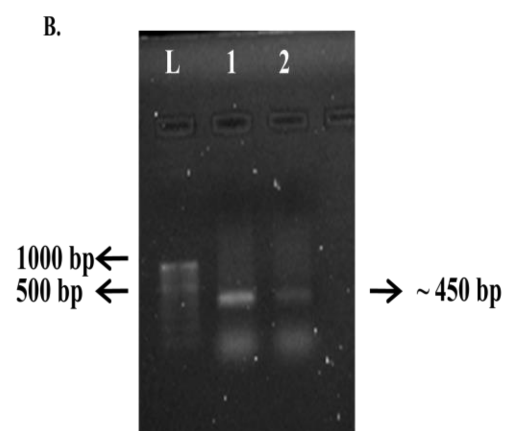


Fig. 4. B. Plasmid PCR outline of selected colony pRGEB32:OsGN1a # G1-4

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G1 @ 60°C annealing temperature, 2 - pRGEB32:OsGN1a # G1 @ 62°C.

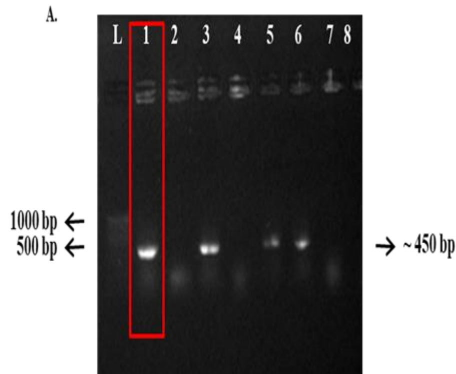


Fig. 5. A. Colony PCR outline of *E. coli* DH5α strain with the insertion of sgRNA

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G2-1, 2 - pRGEB32:OsGN1a # G2-2, 3 - pRGEB32:OsGN1a # G2-3, 4 - pRGEB32:OsGN1a # G2-4, 5 - pRGEB32:OsGN1a # G2-5, 6 - pRGEB32:OsGN1a # G2-6, 7 - pRGEB32:OsGN1a # G2-7, 8 - pRGEB32:OsGN1a # G2-8.

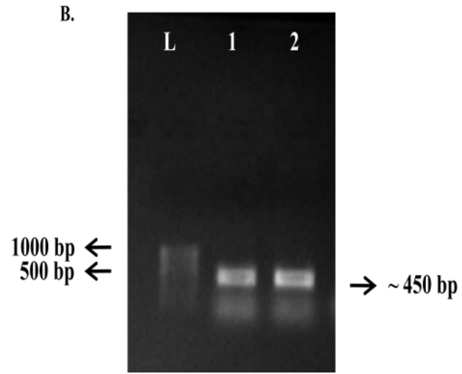
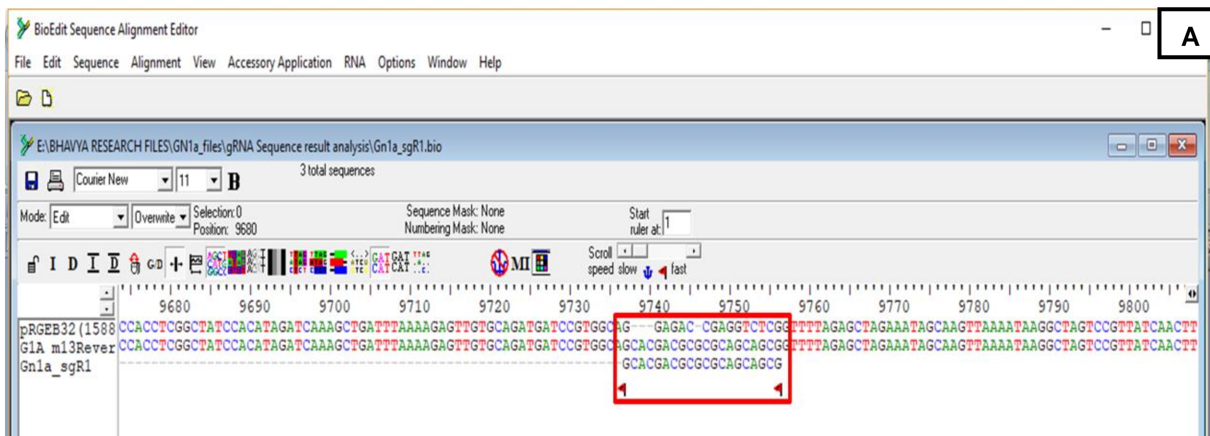


Fig. 5. B. Plasmid PCR outline of selected colony pRGEB32:OsGN1a # G2-1

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G2 @ 60°C annealing temperature, 2 - pRGEB32:OsGN1a # G2 @ 62°C.



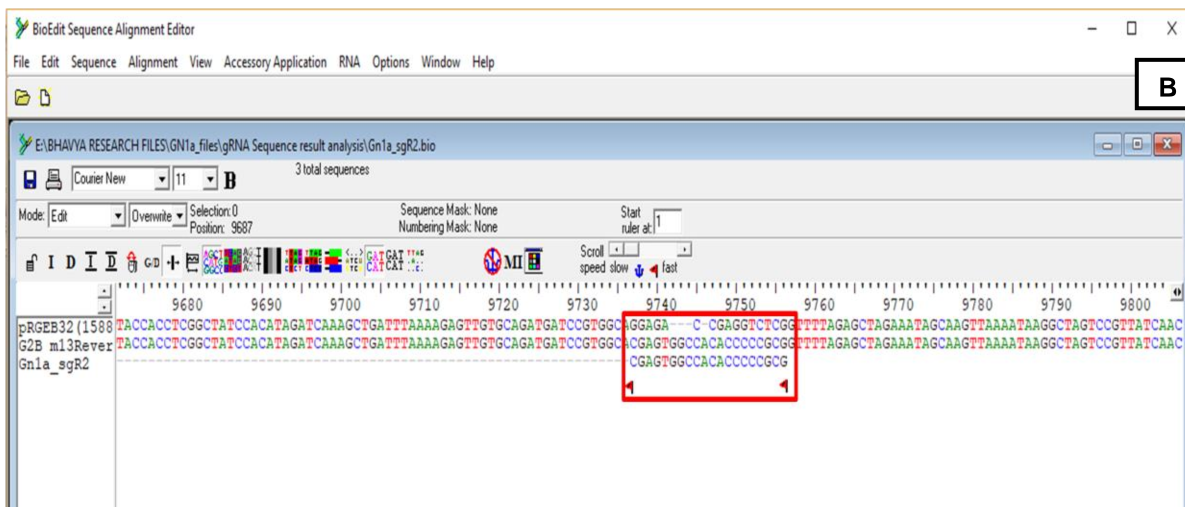


Fig. 6. Sequence alignment showing cloning of sgRNA into pRGEB32 vector backbone

A. pRGEB32:OsGN1a # G1 construct; B. pRGEB32:OsGN1a # G2 construct. BioEdit7.2 software was used for the analysis.

3.6 Mobilization of Positive Clones to *A. tumefaciens* Strain EHA105

The positive clones of CRISPR/Cas9 sgRNA construct of both (OsGN1a#G1 and OsGN1a#G2) for *GN1a* confirmed after sequence analysis were then introduced into *A. tumefaciens* strain EHA105 through freeze-thaw method. After incubation period of 48h at 28° C, ~ 25 and 6 colonies for both sgRNA constructs were observed respectively on LB agar plates containing Rifampicin (25 mg/L), selection marker Kanamycin (50 mg/L).

3.7 Confirmation of Recombinant Vector in *A. tumefaciens* Strain EHA105

The freshly streaked positive clones were verified by colony PCR. Eight colonies for sgRNA1 (pRGEB32:OsGN1a#G1) and six colonies for sgRNA2 (pRGEB32:OsGN1a#G2) were taken for analysis of colony PCR, further colony 4 for sgRNA1 (OsGN1a#G1) and colony 6 for sgRNA2 (OsGN1a#G2) were selected for plasmid isolation and plasmid PCR. Expected band size ~450 bp were observed on 1% agarose gel as shown in Fig 7A and B; Fig 8A and B. To avert false positives confirmed with M13 reverse primer, the respective isolated plasmids of both the sgRNA constructs were analyzed and re-confirmed by plasmid PCR with *hpt* primers that amplify the partial sequence (~ 600 bp) of hygromycin resistant gene in the vector as shown in Fig 9.

Previous studies shown that rice grain yield can be efficiently driven by three characters (i) Number of panicles (ii) Grain number per panicle (GNPP) (iii) Grain weight (Zhou et al., 2018). The improvement of grain number per panicle will increase the overall grain yield. Significantly, the rice grain yield per unit area will be high (Chen et al., 2017; Chen et al., 2018; Zhou et al., 2018). Previous researches have shown that grain yield was governed by over-expressing or suppressing phytohormone related genes. There is growing evidence that plant hormones primarily influence the transcriptional and post-transcriptional regulation of rice genes associated with GNPP in order to mediate the determination of GNPP.

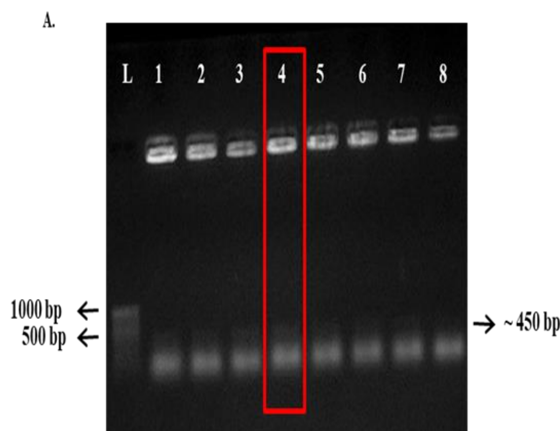


Fig. 7. A. Colony PCR outline of *A. tumefaciens* EHA105 strain with the insertion of sgRNA

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G1-1, 2 - pRGEB32:OsGN1a # G1-2, 3 - pRGEB32:OsGN1a # G1-3, 4 - pRGEB32:OsGN1a # G1-4, 5 - pRGEB32:OsGN1a # G1-5, 6 - pRGEB32:OsGN1a # G1-6, 7 - pRGEB32:OsGN1a # G1-7, 8 - pRGEB32:OsGN1a # G1-8.

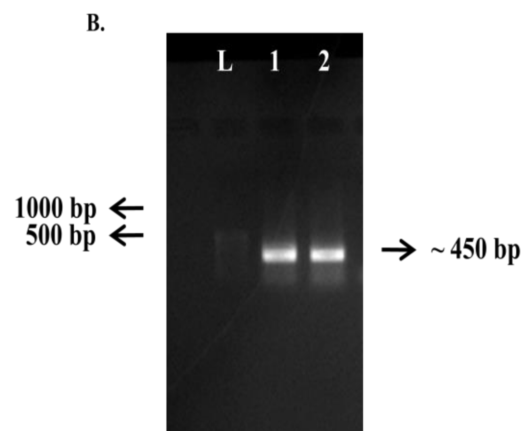


Fig. 7. B. Plasmid PCR outline of selected colony pRGEB32:OsGN1a # G1-4

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G1@ 60°C annealing temperature, 2 - pRGEB32:OsGN1a # G1@ 62°C.

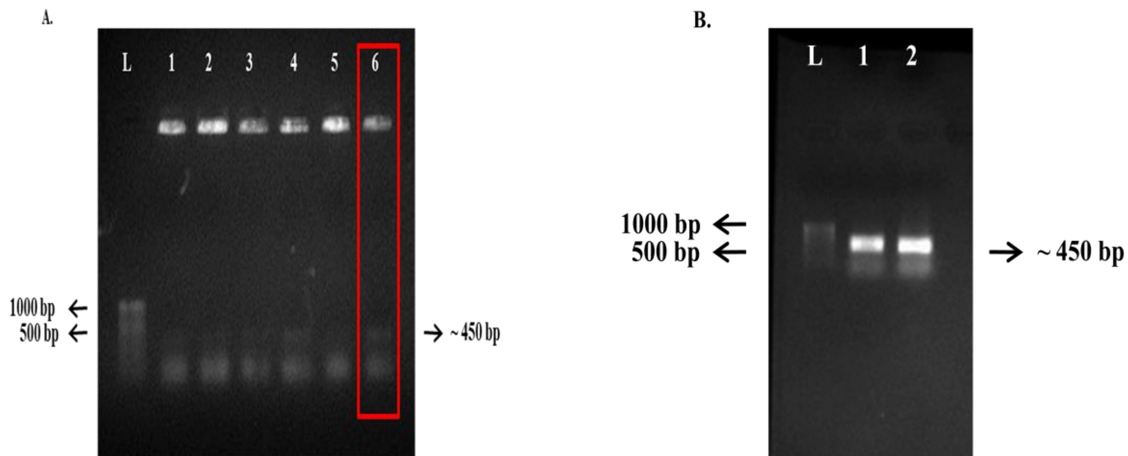


Fig 8. A. Colony PCR outline of *A. tumefaciens* EHA105 strain with the insertion of sgRNA

L - 100bp ladder, 1 - pRGEB32:OsGN1a # G2-1, 2 - pRGEB32:OsGN1a # G2-2, 3 - pRGEB32:OsGN1a # G2-3, 4 - pRGEB32:OsGN1a # G2-4, 5 - pRGEB32:OsGN1a # G2-5, 6 - pRGEB32:OsGN1a # G2-6.

Fig 8.B. Plasmid PCR outline of selected colony pRGEB32:OsGN1a # G2-6

L - 100 bp ladder, 1 - pRGEB32:OsGN1a # G2 @ 60°C annealing temperature, 2 - pRGEB32:OsGN1a # G2 @ 62°C.

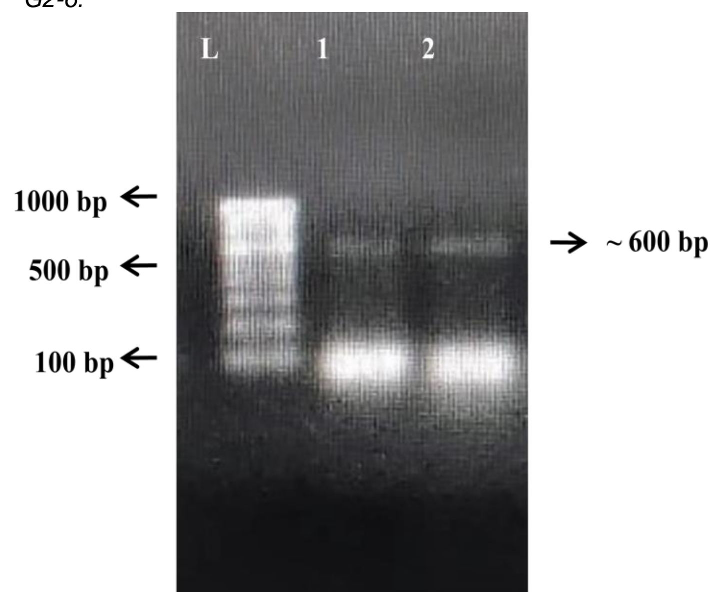


Fig. 9.Plasmid PCR outline of isolated plasmids from transformed *A. tumefaciens* EHA105 using *hpt* primers

L - 100 bp ladder, 1 - pRGEB32:OsGN1a # G1-4, 2 - pRGEB32:OsGN1a # G2-6.

Some of the QTL's positively regulate the GNPP by controlling the Cytokinin concentration and the articulation of *OsCKX2*.*LARGER PANICLE* (*LP*)/*ERECT PANICLE 3* (*EP3*) interacts with SKP1-like protein, when *OsCKX2* expression is up-regulated and CK levels are lowered during rice inflorescence, which increased primary and secondary rachis branch production and grain yield (Li et al., 2011). *DROUGHT AND SALT TOLERANCE* (*DST*), a zinc-finger transcription factor has a negative effect on Cytokinin content, which leads to a lowering in the number of rachis branches and the GNPP in rice. *DST* that modulates the expression of the *OsCKX2*, which encodes Cytokinin oxidase, through a single base insertion that results in a loss of its ability to activate transcription. This change subsequently enhances the rachis branches number, GNPP, and overall yield of grain (Li et al., 2013; Guo et al., 2020).The inactivation of *LONELY GUY*

(*LOG*) led to an early cessation of shoot apical meristem (SAM) function by modulating the levels and spatial arrangement of CK, which reduced both the count of rachis branches and GNPP (Kurakawa et al., 2007).

Some QTL's negatively regulate the GNPP by influencing the Cytokinin levels. *GRAIN AWN DEVELOPMENT1* (*GAD1*) acts as a negative regulator of GNPP, as the GAD1 protein lowers the concentration of cytokinins (CK) by promoting the expression of *DST* and *OsCKX2*. This reduction results in decreased GNPP in wild rice. In cultivated rice, a mutation that alters the codon of GAD1, which disrupts the preserved Cysteine structure, leading to the loss of GAD1 function. Consequently, this enhances GNPP, decreases grain length, and inhibits the development of awns (Jin et al., 2016). *GRAIN NUMBER 1a* (*GN1a*) / *Cytokinin oxidase2* (*OsCKX2*) acts as antagonist to GNPP by decreasing the Cytokinin levels. A decrease in *OsCKX2* expression leads to an increase in Cytokinin levels within the inflorescence meristem, which promotes the development of additional rachis branches and GNPP, ultimately enhancing grain yield (Ashikari et al., 2005) and resistance to lodging (Tu et al., 2022).

Plant vector pRGEB32 is the most pre-dominant binary vector system exploited for site-directed genome editing using CRISPR/Cas9 system, the efficient tool reported for precise mutagenesis in most crop species (Xie et al., 2014). The vector system has sgRNA cloning sites fringed with *Bsa*I restriction sites, driven by pol III type promoter of rice U3 snoRNA. Cas9 gene from *Streptococcus pyogenes* attached with nuclear-localizing signal (NLS), encodes for Cas9 nuclease (ribonucleoprotein), that directs DNA-targeted cleavage, whose expression driven by rice ubiquitin promoter. Kanamycin (Kan^R) and Hygromycin (Hyg^R) resistant genes as bacterial and plant selection markers respectively influenced by CaMV35S promoter.

The CRISPR/Cas9 recombinant cassettes were constructed by ligating the phosphorylated, annealed sgRNA oligos in pRGEB32 vector and then cloned into *E. coli* DH5α strain by heat-shock method @ 42°C for 90s(Chang et al., 2017). The cloning of this cassette poses a challenge because of larger plasmid size (~ 15.9kb) affecting the uptake of plasmid, possibly resulting in lesser number of bacterial transformants. Despite this expected hurdles, we obtained more than 50 transformants, among which 8 were selected for further sub-culturing. The confirmation of positive clones was done by colony PCR, followed by plasmid isolation and plasmid PCR. PCR positive clones were analyzed by sequence alignment after sequencing.

The positive clones were then mobilized into *A. tumefaciens* EHA105 with freeze-thaw method @ 37°C for 5min (Holsters et al., 1978; Weigel and Glazebrook, 2006). Approx 6 - 20 colonies were observed in OsGN1a#G1 and OsGN1a#G2. *Agrobacterium tumefaciens*-mediated transformation became an adaptable practice in lab conditions (Chan et al., 1993 ; Hiei et al., 1994; Slamet-Loedin et al., 2014) , for its ideal capability to deliver a desired DNA fragment from a plasmid into a host plant (Gelvin, 2010), enables efficient integration of un-rearranged, one-copy DNA, which end up with stable expression than many gene copies or scrabbled inserts which will lead to unwanted mutation (Iglesias et al., 1997).

4. CONCLUSION

Yield is considered to be the significantly important physiological trait governed by many QTL's, some of which are negatively influencing the yield. In this study, CRISPR/cas9 technique, a genome editing tool used for site-targeted editing of *GRAIN NUMBER 1a* gene, which negatively regulates the yield by degrading Cytokinin. Precisely efficient sgRNAs were designed using CRISPR-P v2.0 software, synthesized as sense and antisense strands with *Bsa*I restriction sites by IDT, phosphorylated, annealed, ligated into pRGEB32 vector, then transformed to *E. coli* DH5α and then transferred to *A. tumefaciens* EHA105.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc. have been used during the writing or editing of manuscripts.

Details of the AI usage are given below:

1. Grammarly: Free AI Writing Assistance
2. ChatGPT Open AI
3. Quillbot

FUTURE SCOPE

The present study could be helpful for meeting the food demand for growing population and also

for the farmer's welfare by increasing the yield through targeted genome editing using CRISPR/cas9 system.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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