Original Paper

Efficient genome editing and gene knockout in *Setaria viridis* with CRISPR/Cas9 directed gene editing by the non-homologous end-joining pathway

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Abstract The CRISPR/Cas9 system has been used for genome editing in several organisms, including higher plants. This system induces site-specific mutations in the genome based on the nucleotide sequence of engineered guide RNAs. The complex genomes of C4 grasses makes genome editing a challenge in key grass crops like maize (*Zea mays*), sorghum (*Sorghum bicolor*), *Brachiaria* spp., switchgrass (*Panicum virgatum*), and sugarcane (*Saccharum* spp.). *Setaria viridis* is a diploid C4 grass widely used as a model for these C4 crop plants. Here, an optimized CRISPR/Cas9 binary vector that exploits the non-homologous end joining (NHEJ) system was used to knockout a *green fluorescent protein* (*gfp*) transgene in *S. viridis* accession A10.1. Transformation of embryogenic callus by *A. tumefaciens* generated ten glufosinate-ammonium resistant transgenic events. In the T0 generation, 60% of the events were biallelic mutants in the *gfp* transgene with no detectable accumulation of GFP protein and without insertions or deletions in predicted off-target sites. The *gfp* mutations generated by CRISPR/Cas9 were stable and displayed Mendelian segregation in the T1 generation. Altogether, the system described here is a highly efficient genome editing system for *S. viridis*, an important model plant for functional genomics studies in C4 grasses. Also, this system is a potential tool for improvement of agronomic traits in C4 crop plants with complex genomes.

Key words: CRISPR/Cas9, genome editing, non-homologous end-joining, panicoid grasses, *Setaria viridis*, stable genetic transformation.

Introduction

Genome editing is a powerful tool to introduce deletions, insertions, or sequence modifications into organismal genomes. Genome editing with the <u>C</u>lustered <u>R</u>egulatory <u>I</u>nterspaced <u>S</u>hort <u>P</u>alindromic <u>R</u>epeat-associated endonuclease protein 9 (CRISPR/Cas9) system has been optimized for a range of organisms, replacing other site-specific nucleases (*e.g.*, Meganucleases, ZNFs, and TALENs) due to its greater simplicity, specificity, and efficiency (Cong et al. 2013; Jinek et al. 2012; Makarova et al. 2015; Mali et al. 2013; May et al. 2013; Zhang 2013). The CRISPR/Cas9 system creates double-stranded DNA breaks (DSBs) at a specific target sequence specified by the sequence of a guide RNA (gRNA). The break is repaired by either the non-homologous end joining (NHEJ) pathway, which generates nucleotide insertions or deletions (indels) at the target site, or the homology-

directed repair (HDR) pathway, which can generate single and double knock-ins of a desired target gene or promoter sequence (Li et al. 2016; Sun et al. 2016; Zhao et al. 2016). The NHEJ pathway is more efficient than the HDR pathway (Li et al. 2016; Liang et al. 2017; Sun et al. 2016; Tang et al. 2017; Woo et al. 2015; Zhang et al. 2016). However, the efficiency of either CRISPR/Cas9 system varies between phylogenetically distant plant species. Thus, it is necessary to optimize the elements of the CRISPR/Cas9 system for the target genome (Basso et al. 2020; Li et al. 2016; Yin et al. 2017).

Several features of the C4 grass *Setaria viridis* make it an experimental model plant for C4 grass crops, including a short life cycle, high seed production, and established protocols for genetic transformation and plant regeneration (Brutnell et al. 2010; Li and Brutnell 2011; Martins et al. 2015), as well as a small diploid genome that is fully sequenced (Bennetzen et al. 2012). This model plant is widely used to study C4 grass functional genomics and for proof-of-concept experiments for panicoid bioenergy feedstocks and food crops with highly complex genomes like maize (*Zea mays*), sorghum (*Sorghum bicolor*), *Brachiaria* spp., switchgrass (*Panicum virgatum*), and sugarcane (*Saccharum* spp.).

Huang et al. (2019) edited the genome of *S. viridis* with a CRISPR/Cas9 genome system to introduce mutations in the *SvLes1* (Sevir.5G085400) gene. More recently, Weiss et al. (2020) demonstrated efficient genome editing in *S. viridis* with a combination of a CRISPR/Cas9 and expression of exonuclease Trex2 (CRISPR/Cas9_Trex2), which promoted repair by either NHEJ or microhomology-mediated end joining (MMEJ). These authors showed that this multiplex CRISPR/Cas9_Trex2 system induced targeted *indels* in both the *svDrm1a* and *svDrm1b* genes of T0 transgenic events at a frequency of 73% to 100%. These *indels* were transmitted to at least 60% of the transgene-free T1 plants, with 33% of them containing biallelic or homozygous *indels* in both genes.

Here, we optimized a CRISPR/Cas9 binary vector that promotes the activity of the NHEJ pathway and this vector was used to edit a transgenic copy of the *green fluorescent protein* (*gfp*) gene in *S. viridis* accession A10.1. This study generated ten biallelic mutants (homozygous) at the T0 generation. These *indels* in the *gfp* transgene were stable and showed Mendelian segregation in the T1 generation. These lines carried no off-target *indels* in predicted off-target sites, indicating highly site-specific targeting by this combination of CRISPR/Cas9 binary vector and gRNA.

Materials and methods

Construction of a CRISPR/Cas9 binary vector to enhance genome editing through NHEJ pathway

The T-DNA of the binary vector is composed of (*i*) selection marker gene (*bar* gene from *Streptomyces hygroscopicus* for resistance to glufosinate-ammonium herbicide), with an intron sequence, controlled by *Oryza sativa* constitutive promoter *actin 1* (*Os*Act-1), and 3′rbcsE9 transcription terminator; (*ii*) Cas9 nuclease from *Streptococcus pyogenes* (*Sp*Cas9WT), with codon-usage optimized for monocots and fused in-frame with two SV40 nuclear localization signal (NLS) at the 5′ and 3′ end of the gene. This fragment was engineered under control of the constitutive ubiquitin-1 promoter from *Zea mays* (*Zm*UBI-1), with an intron sequence and linked with the nopaline synthase (*NOS*) transcription terminator sequence. The Kozak sequence CCG AA (optimized Kozak sequence for monocots) was introduced in front of the translation initiation site (ATG); (*iii*) DNA sequence that corresponds to the gRNA sequence under control of the constitutive U6-2 small nuclear RNA promoter (coding for RNA polymerase III) from *Oryza sativa* (*Os*U6), with extra guanine (G) nucleotide at 3′ end fused in-frame at the 3′ end of a synthetic RNA sequence (scaffold RNA used for Cas9 coupling). The dyad symmetry sequence was added after the transcription stop signal (poly A7). The gRNA was designed for targeting the 5′ end of the *gfp* (*green fluorescent protein*) gene and direct the cleavage by Cas9 from the sense strand of target DNA using NGG sequence as the Protospacer Adjacent Motif (PAM). Thus, the *gfp* transgene was used as target DNA to optimize the CRISPR/Cas9 NHEJ system in *S. viridis* and the GFP protein as a reporter of the knockout efficiency. The absence of off-target mutations was analyzed by scanning the Phytozome 12 *S. viridis* genome (*Setaria viridis* v2.1, DOE-JGI, (http://phytozome.jgi.doe.gov/) using GT-Scan (O'Brien and Bailey 2014) and CCTop (Stemmer et al. 2015) software. The binary vector also contained the CoIE1 and pVS1 origin of replication from *Escherichia coli* and *Agrobacterium tumefaciens*, respectively, and resistance marker gene to streptomycin/spectinomycin (*Sm/Sp*). The CRISPR/Cas9 binary vector was synthesized, and assembled by DNA Cloning Service (www.dna-cloning.com; Hamburg, Germany).

Stable transformation and regeneration of Setaria viridis gene editing events from embryogenic callus

S. viridis (accession A10.1) was co-transformed by a high-efficiency *Agrobacterium*-mediated transformation protocol (Martins et al. 2015). After removing the lemmas and paleas, mature seeds were disinfested, blotted onto sterile filter paper, and transferred to callus induction medium (CIM2) for embryogenic callus induction. After 22 days of incubation in the dark at 25±2°C, embryogenic calli were isolated and transferred onto a fresh CIM2 medium. After a week, selected embryogenic calli were transformed by *A. tumefaciens* EHA105. Embryogenic calli from a homozygous

transgenic *S. viridis* plants constitutively expressing GFP and resistant to hygromycin B (30 mg/l) (Martins et al. 2015) were co-transformed with the CRISPR/Cas9 NHEJ binary vector. Selection with glufosinate-ammonium herbicide (3 mg/l; Liberty®-Bayer CropScience) identified ten independent CRISPR/Cas9 NHEJ events after. Transgenic seedlings were planted into pots containing 250 g of latosoil, substrate (Plantmax®), and vermiculite (Agrifloc, Brasil Minérios) mixture (3 : 1 : 0.5; w/w/w). The plants were maintained in a growth chamber under 16/8-h photoperiod at $500 \mu mol\,m^{-2}\,s^{-1}$ light intensity, $26 \pm 2°C$ temperature, and 65% relative humidity until seed establishment (seeds from T1 generation).

Genotyping of the gene editing events

Genomic DNA was isolated from co-transforming T0 lines and control (GFP positive, but not co-transformed with our CRISPR/Cas9 binary vector) plants using the CTAB method (Doyle and Doyle 1987). PCR assays were carried out using specific primers targeting *bar* gene and *Zm*UBI-1:GFP to confirm the insertion of *Zm*UBI-1:GFP and CRISPR/Cas9 transgene in the regenerated plants from selection with glufosinate-ammonium herbicide. PCR-positive events were also confirmed for BAR protein accumulation with a quick test strip (QuickStix™ Kit for PAT/bar, EnviroLogix, Inc., USA), according to the manufacturer's instructions. The *Zm*UBI-1:GFP PCR products (1.23 kb length) from ten co-transforming events and two control plants were directly purified and dual sequenced (forward and reverse) using the Sanger method by Macrogen Service (Geumcheon-gu, Seoul, South Korea). Chromatograms were analyzed using Geneious R10 software (Kearse et al. 2012). Mono (heterozygous) and biallelic (homozygous) *indels* in the DNA sequence targeted by the gRNA were decoded using standard degenerate symbols (IUPAC/IUB), based on the color peaks of the chromatograms, according to the Degenerate Sequence Decoding (DSD) method (Ma et al. 2015). Chromatograms from both forward and reverse sequencing confirmed the *indels* and their exact positions in the target sequence. The inheritance and stability of *indels* induced by the CRISPR/Cas9 system were confirmed by PCR, and subsequent Sanger sequencing of the *Zm*UBI-1:GFP transgene from EC1 to EC3, EC5, and EC7 events from T1 generation. Wild-type plants (ECWT) were used as control. The T-DNA copy number in each event was estimated using seeds from T1 co-transforming events. One hundred seeds were grown in a selective MS medium containing 5 mg/l glufosinate-ammonium. Ten-day-old resistant and sensitive plants were counted and analyzed statistically using the χ-Squared test. Events with a 3 : 1 Mendelian segregation ratio were considered as single T-DNA insertion. The occurrence of putative off-targets was screened by Sanger sequencing of PCR product from the top two predicted off-target sites, which have respectively 1 and 2 nucleotide mismatches in the core sequence, besides 3 and 2 nucleotide mismatches farther from the PAM sequence, compared with the sequence of gRNA. Highly pure DNA samples from co-transforming T0

transgenic events named EC1 to EC5 and control plant ECWT1 were amplified by PCR using primers flanking the predicted off-target sites. PCR products were previously checked using agarose gel electrophoresis, purified using a commercial kit, and sequenced by the Sanger method in a forward direction using the same primers.

Relative expression of gfp mRNA in gene editing events

Total RNA was isolated from leaf tissue of *S. viridis* adult plants (30 days after germination), with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA concentration was estimated using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Massachusetts, USA). RNA integrity was analyzed in 1% agarose gel electrophoresis. RNA samples were treated with RNase-free RQ1 DNase I (Promega), according to the manufacturer's instructions. Then, $2 \mu g$ of DNase-treated RNA were used as the template for cDNA synthesis using oligo(dT)18 primer and SuperScript III RT (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA produced was quantified by spectrophotometry and diluted with nuclease-free water to $200\,ng/\mu l$. Quantitative reverse transcription PCR (qRT-PCR) assays were carried out in ABI StepOne Plus Real-Time PCR System (Applied Biosystems, USA). The reactions were performed using 300 ng cDNA, $0.2 \mu M$ of each gene-specific primer, and Platinum™ SYBR™ Green qPCR SuperMix-UDG w/ROX (Invitrogen). Statistical difference between co-transforming EC1 to EC10 events and *S. viridis* A10.1 (wild-type) was confirmed by Tukey's HSD test (p-value<0.01) using the SASM-Agri statistical package (Canteri et al. 2001). All reactions were performed with three technical replicates for each sample and conditions, according to Martins et al. (2016). The relative expression was calculated using the $2^{-\Delta Ct}$ method (Schmittgen and Livak 2008) using *Sv*SUI (Sevir.2G348300) as an endogenous reference gene. All primers used in the present work are listed in Supplementary Table S2.

GFP protein detection in gene editing events

GFP-specific fluorescence in leaves of the co-transforming events was visualized in Leica M205 FA stereomicroscope equipped with either a long pass filter with a 395–455 nm excitation filter and a 480 nm emission filter or a GFP filter with a 450–490 nm excitation filter and a 500–550 nm emission filter. Detection of GFP protein in the crude protein extracts isolated from leaf tissue of adult co-transforming and transgenic plants was carried out using indirect ELISA (Clark 1981). Initially, the protein amount in the total protein extracts was quantified using Bradford's method (Bradford 1976). Serological assays were conducted using the monoclonal IgG1 Anti-GFP (G1546, Sigma-Aldrich) as a primary antibody produced in mice, which were immunized with a synthetic peptide corresponding to amino acids 132–144 of GFP. Polyclonal anti-mouse IgG produced in goat and alkaline phosphatase-conjugated (A3688,

Sigma-Aldrich) was used as a secondary antibody. Samples consisting of leaves from *S. viridis* A10.1 non-transgenic plants (wild-type plant used as a negative control for GFP in the indirect ELISA), transgenic ECWT1 and ECWT2 plants (GFP-positive control for the indirect ELISA), and co-transforming EC1 to EC10 events were grounded to a fine powder with pestle and mortar in liquid nitrogen and homogenized in 1:2 (w/v) extraction buffer (10 mM Na_2CO_3, 10 mM $NaHCO_3$, 15 mM NaN_3, 5 mM Na-DIECA, 0.2% bovine serum albumin, 2% PVP40, pH 9.6). Plant samples were considered GFP-positive when absorbance at 405 nm was at least twice the average value of the negative control.

Results

We constructed a new CRISPR/Cas9 binary vector engineered with several genetic elements to enhance the efficiency and specificity of genome editing in *S. viridis*, as described in Material and Methods and shown in Figure 1a. We also designed a gRNA to target the 5′-
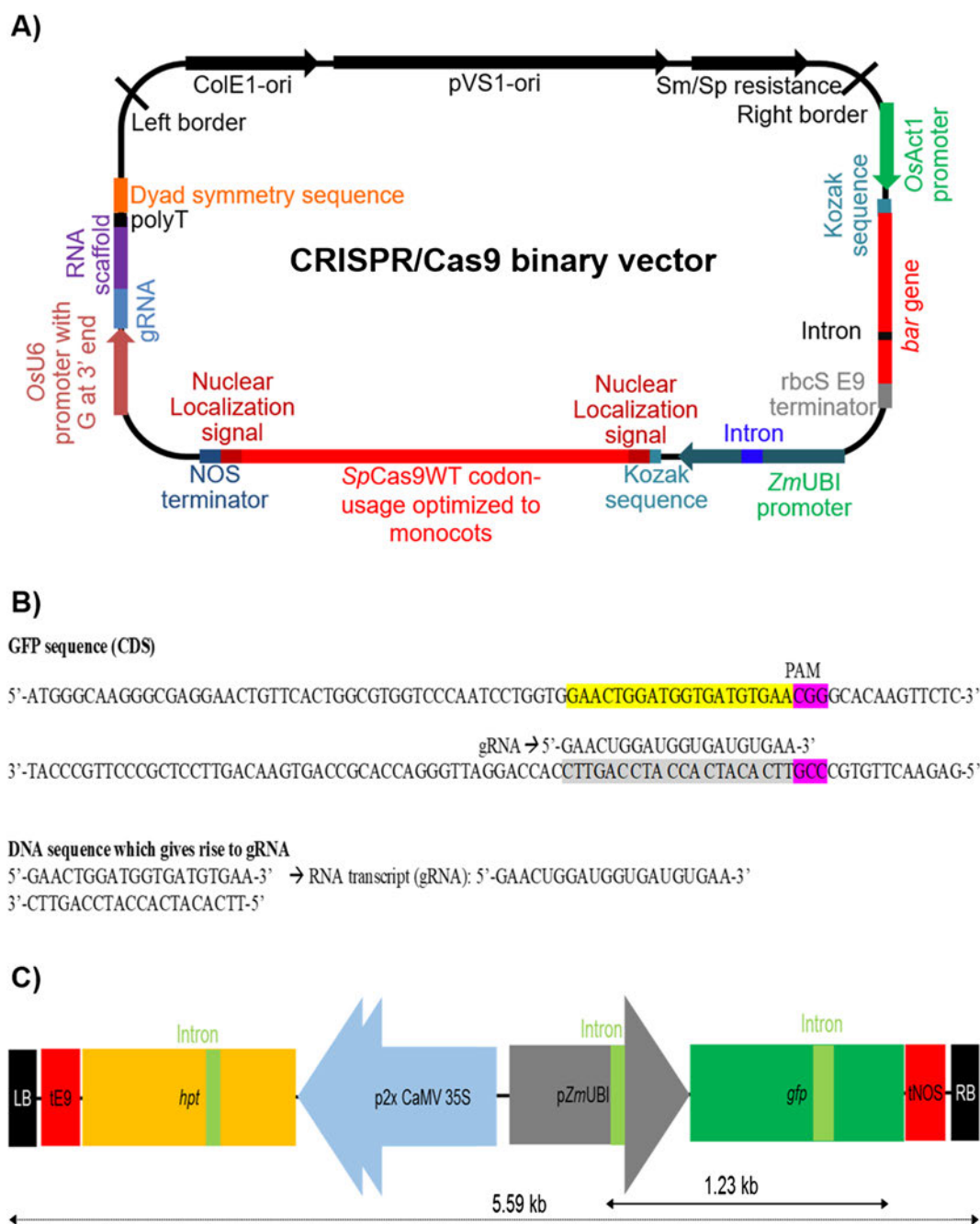
Figure 1. Genome editing and gene knock-out strategy using the CRISPR/Cas9 system exploring the non-homologous end joining (NHEJ) pathway in *S. viridis* accession A10.1. (a) Optimized CRISPR/Cas9 NHEJ binary vector for genome editing in *S. viridis*; (b) schematic diagram of the guide RNA targeting the *gfp* gene. (c) Schematic T-DNA region of the binary vector used to transform *Setaria viridis* for constitutive expression of GFP protein. The generated *Setaria viridis* events were edited in co-transforming events by CRISPR/Cas9 system.

Table 1.   Sequences of the mono and biallelic *indels* in *gfp* transgene induced by CRISPR/Cas9 NHEJ system, *gfp* mRNA expression level, GFP protein accumulation, and segregation behavior of the CRISPR/Cas9 transgene in gene edited plants from events EC1 to EC10 compared to parental unedited *gfp* transgenic plants (ECWT1 and ECWT2).

Events	Decoding of mono and biallelic mutations	Decoding results	*gfp* mRNA accumulation	GFP protein accumulation	Segregation ratio in T_1
ECWT1	GGATGGTGATGTGAACGGGCAC	Non-edited control lineage 1	Positive	Positive	—
ECWT2	GGATGGTGATGTGAACGGGCAC	Non-edited control lineage 2	Positive	Positive	—
EC1	ECWT line GGAACTGGATGGTGATGTGAACGGGCAC- EC1 line GGAACTGGATGGTGAT-KKRAMSGGCMCM Allele 1 GGAACTGGATGGTGATTGAACGGGCACA ****************_*********** Allele 2 GGAACTGGATGGTGATGTGAACGGGCAC **************************	Allele 1: deletion of G nucleotide; Allele 2: no indels; Monoallelic mutant.	Positive	Positive	3:1
EC2	ECWT line GGAACTGGATGGTGATG-TGAACGGGCAC EC2 line GGAACTGGATGGTGATGWWSMRSSRSMA- Allele 1 GGAACTGGATGGTGATGATGAACGGGCA- *****************_**********- Allele 2 GGAACTGGATGGTGATGT--ACGGGCACAA *********************_-***********	Allele 1: insertion of A nucleotide; Allele 2: deletion of GA nucleotides; Biallelic mutant.	Positive	Negative	3:1
EC3	**Chromatogram from forward sequencing** ECWT line GGAACTGGATGGTGATGGTGATGTGAACGGGCACAAGT EC3 line GGAACTGKWYRSWR----GWWCKSSSWCARSK Allele 1 GGAACTGGATGGTG----GAACGGGCACAAGT ****************____**********??? Allele 2 GGAACTG-TT-CACAAGTTCTCCGTCACAA ********__-__* **-* ****** **Chromatogram from reverse sequencing** ECWT line GACGGGAGAACTTGTGCCCGTTCACATC-ACCA-T-CC EC3 line GACGGGAGAACTTGTGMMCRKTY-CA-CMWCSAKTKSS Allele 1 GACGGGAGAACTTGTGCCCGTTC-CA-CCACCATTGCC ****************_**-*_****-*_** Allele 2 GACGGGAGAACTTGTGAA--CAGTTCACATCGA--GTT-CAGTTCC ********__----*_*********----*_*----* *_********	Allele 1: deletion of ATGT nucleotides; Allele 2: two deletions of G nucleotide; Biallelic mutant. Allele 1: deletions of A and T nucleotides and insertions of C/A, T and G nucleotides; Allele 2: insertions of AA, A, G, and G nucleotides and two deletions of CC nucleotides; Biallelic mutant.	Positive	Negative	3:1
EC4	ECWT line GGAACTGGATGGTGATGGTGATGTGAACGGGCACAAG EC4 line GGAACTGGATGGTGAT-KKARMRSGSRCAMR Allele 1 GGAACTGGATGGTGAT-TGAACGGGCACAAG ****************_********** Allele 2 GGAACTGGATGGTGATGTGATGTAGAACGGGCACA ****************_*-**********	Allele 1: deletion of G nucleotide; Allele 2: insertion of A nucleotide; Biallelic mutant.	Positive	Negative	3:1
EC5	ECWT line GGAACTGGATGGTGATGGTGATGTGAACGGGCACAAG EC5 line GGAACTGGATGGTGAT-KRAMCRSSMMCAMK Allele 1 GGAACTGGATGGTGAT-TGAACGGGAACAAG ****************_********** Allele 2 GGAACTGGATGTGAT--GAACGGGCACA ****************_-***********	Allele 1: deletion of G nucleotide; Allele 2: deletions of GT nucleotides; Biallelic mutant.	Positive	Negative	3:1

Table 1. Continued.

Events	Decoding of mono and biallelic mutations	Decoding results	*gfp* mRNA accumulation	GFP protein accumulation	Segregation ratio in T_1

EC6	ECWT line GGAACTGGATGGTGATGTGATGTGAACGGGCACAA
	EC6 line GGAACTGGATGGTGATGTGATG--WWSRRSSRSMA
	Allele 1 GGAACTGGATGGTGATG--AACGGGCACAA
	  ************-_*********
	Allele 2 GGAACTGGATGGTGATGTTGAACGGGCA
	  **********************_**********
		Allele 1: deletion of TG nucleotides;
		Allele 2: one insertion of T nucleotide;
		Biallelic mutant.
			Positive	Negative	3 : 1

EC7	ECWT line GGAACTGGATGGTGATGTGATGTGAACGGGCAC
	EC7 line GGAACTGGATGGTGATGTGATGWRAMSGGSMMM
	Allele 1 GGAACTGGATGGTGATGTGATGTGAACGGGCAC
	  *********************************
	Allele 2 GGAACTGGATGGTGATG--AACGGGCACA
	  *****************--_**********
		Allele 1: no indels;
		Allele 2: deletion of TG nucleotides;
		Monoallelic mutant.
			Positive	Positive	3 : 1

EC8	ECWT line GGAACTGGATGGTGATGTGATGTGAACGGGCACA
	EC8 line GGAACTGGATGGTGATGTGATGKRAMSGGSMMM
	Allele 1 GGAACTGGATGGTGATG-GAACGGGCACA
	  *******************-_*********
	Allele 2 GGAACTGGATGGTGATGTGTGAACGGGCAC
	  ******************************
		Allele 1: deletion of T nucleotide;
		Allele 2: no indels;
		Monoallelic mutant.
			Positive	Positive	3 : 1

EC9	ECWT line GGAACTGGATGGTGATGTGATGTGAACGGGCACAC
	EC9 line GGAACTGGATGGTGATGTGATGTGATGWRMRSGSRCMM
	Allele 1 GGAACTGGATGGTGATGTGAACGGGCACAC
	  ***************************
	Allele 2 GGAACTGGATGGTGATG--AACGGGCACAC
	  *****************--_**********
		Allele 1: no indels;
		Allele 2: deletion of TG nucleotides
		Monoallelic mutant.
			Positive	Positive	3 : 1

EC10	ECWT line GGAACTGGATGGTGATGTGATGTGAACGGGCACAC
	EC10 line GGAACTGGATGGTGATGTGATGATGRAMSGGSACMA
	Allele 1 GGAACTGGATGGTGATG--AACGGGCACAC
	  *****************-_*********
	Allele 2 GGAACTGGATGGTGATG-GAACGGGACCAC
	  *****************-_*********
		Allele 1: deletions of TG nucleotides;
		Allele 2: deletion of T nucleotide;
		Biallelic mutant.
			Positive	Negative	3 : 1

end of a *gfp* transgene (Figure 1b) previously inserted into the genome of *S. viridis* (Figure 1c), directing Cas9 cleavage of the sense strand of the target site using NGG nucleotides as the PAM sequence.

Stable genetic transformation and genotyping of recovered genome editing events

Embryogenic calli from transgenic plants overexpressing *gfp* were transformed with the engineered CRISPR/

Cas9 binary vector to generate *indels* that disrupt the *gfp* transgene (Figure 2a). Ten independent transgenic events obtained following *Agrobacterium*-mediated transformation were sequentially named as EC1 to EC10. PCR analysis confirmed these lines carried the CRISPR/Cas9 minimal expression cassette (Figure 2b and 2c), which was confirmed by the presence of the BAR protein from the *bar* gene (Table 2). Plants from these events were compared to two independent *gfp* overexpressing
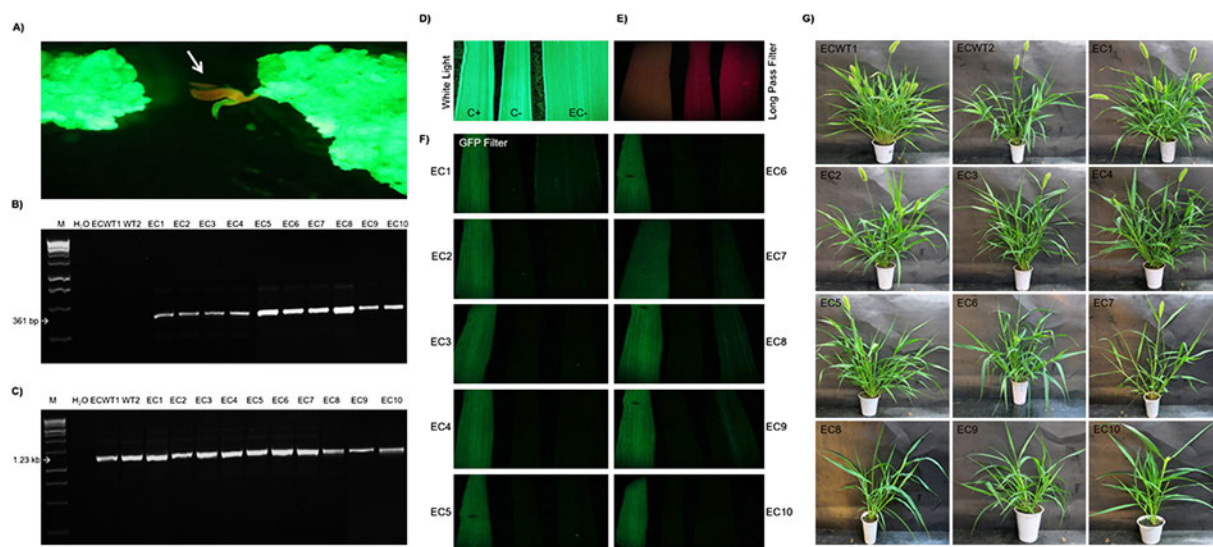

Figure 2.   Genetic transformation of *Setaria viridis* with CRISPR/Cas9 NHEJ binary vector and plant evaluation. (a) Embryogenic callus observed under GFP long-pass filter (excitation and emission wavelength 395–455 nm and 480 nm, respectively). Arrowhead indicates a transgenic shoot regenerated under glufosinate-ammonium selection. (b) Agarose gel electrophoresis of PCR products from CRISPR-edited plants using primers specific for *bar* gene (top) and *Zm*UBI-1:GFP (bottom). MWM=1 kb ladder DNA molecular weight marker; ECWT1 and ECWT2 are transgenic plants positive for *Zm*UBI-1:GFP and negative for CRISPR/Cas9 NHEJ minimal expression cassette; EC1 to EC10 are independent co-transforming and edited events. (c) Imaging using GFP Filter (excitation and emission wavelength 450–490 nm and 500–550 nm, respectively). Leaf from *S. viridis* A10.1 constitutively expressing GFP protein (positive control: C+); leaf from non-transgenic plants (negative control: C−); leaves from *S. viridis* A10.1 constitutively expressing GFP protein and co-transformed with CRISPR/Cas9 NHEJ binary vector (EC1 to EC10). (d) Phenotype of mature edited T0 event (EC1) compared with a non-edited plant (ECWT1 and ECWT2), illustrating the normal development of edited plants.

Table 2.   Results of indirect ELISA assays (absorbance values at 405 nm) and quick test strip to serological detection, respectively, of GFP and BAR proteins in leaf samples from co-transforming events of *Setaria viridis*. Primary antibody against the C-terminal portion was used for GFP detection, while for BAR protein detection was used the QuickStix™ Kit for PAT/bar (EnviroLogix, Inc., USA).

Sample	Absorbance values at 405 nm	ELISA for GFP	Quick test strip for BAR
Buffer	0.101	Negative	Negative
SvA10.1 (negative control; wild-type plants)[a]	0.332	Negative	Negative
ECWT1 (GFP-positive control 1)[b]	1.372	Positive	Negative
ECWT2 (GFP positive control 2)[c]	1.624	Positive	Negative
EC1	0.762	Positive	Positive
EC2	0.412	Negative	Positive
EC3	0.358	Negative	Positive
EC4	0.421	Negative	Positive
EC5	0.456	Negative	Positive
EC6	0.309	Negative	Positive
EC7	0.695	Positive	Positive
EC8	0.869	Positive	Positive
EC9	0.987	Positive	Positive
EC10	0.379	Negative	Positive

a=wild-type *Setaria viridis* A10.1 (non-transgenic; negative control for GFP in the indirect ELISA); b and c=transgenic plants positive control for *Zm*UBI-1:GFP and negative control for CRISPR/Cas9 NHEJ (used as positive controls for GFP protein in the indirect ELISA); EC1 to EC10=co-transforming events PCR-positive to *Zm*UBI-1:GFP and CRISPR/Cas9 NHEJ minimal expression cassette.

plants (GFP-positive control plants, named ECWT1 and ECWT2) and two non-transgenic wild-type plants (GFP-negative control plants). GFP accumulation in leaves of young gene edited plants, detected by fluorescence microscopy, presented three accumulation patterns: (*i*) absence, (*ii*) low GFP accumulation, and (*iii*) GFP accumulation equivalent to ECWT1 or ECWT2 control plants (Figure 2c). The results indicate different levels of allele disruption in each event. The morphology of adult T0 gene edited plants appeared similar to control plants, indicating transformation with the CRISPR/Cas9 binary vector did not cause pleiotropic alterations of growth and development (Figure 2d). The CRISPR/Cas9 minimal expression cassette segregated at a 3 : 1 ratio in the T1 progeny of events EC1 to EC10, indicating a single copy insertion for each event, as expected for *A. tumefaciens*-mediated transformation.

To identify the nature of the mutation introduced by gene editing in each event, purified PCR products amplified from the targeted genomic region in the T0 EC1-EC10 and GFP-positive control plants (ECWT1 or ECWT2) were sequenced by Sanger sequencing (Figure 3a). Sequencing revealed the presence of *indels* at the gRNA-targeted site in the *gfp* transgene of plants from each of the ten events and these changes appeared as either mono and biallelic *indels* (Table 1, Figure 3B).

Finally, the effect of these mutations on *gfp* expression and GFP protein accumulation in the adult T0 plants was determined with RT-qPCR and indirect ELISA assays, respectively. mRNA from the *gfp* transgene was observed in all plants from all events (Figure 3C), while GFP protein levels were different amongst the lines, ranging from low accumulation in plants with monoallelic mutations to no accumulation in plants with biallelic mutations (Table 2). These results indicate the mutations introduced by gene editing modified the mRNA sequence to encode either a truncated protein (resulting from a premature stop codon) or a protein with an amino acid sequence different from GFP (resulting from a frameshift mutation) (Supplementary File S1).

Inheritance of introduced indels and the absence of off-target mutations in recovered events

Previous studies in other plant species show that the mutations induced by the CRISPR/Cas9 system are stable and heritable as classical Mendelian alleles. Similarly, the mutations present in the T0 edited plants where heritable in the T1 generation and segregated as expected (Supplementary Figure S1A). Because CRISPR/Cas9 can target off-target sequences, we evaluated the specificity of our CRISPR/Cas9 system by sequencing the two genome locations that best matched the gRNA sequence
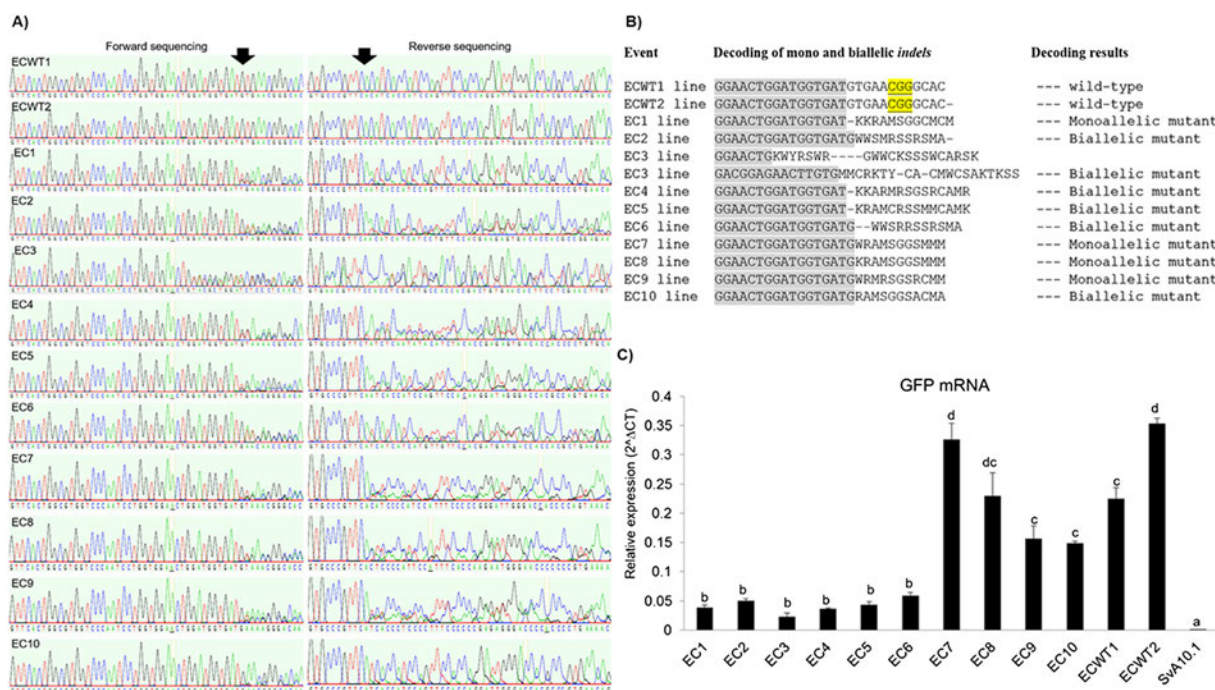

Figure 3. Gene editing and gene expression. (a) Sanger sequencing of PCR products from target sequence of the CRISPR/Cas9 NHEJ system. Forward and reverse sequencing were carried out using *Zm*UBI(F) and GFP(R) primers (Supplemental Table S2). ECWT1 and ECWT2 are transgenic plants positive for *Zm*UBI:GFP plants and negative for CRISPR/Cas9 NHEJ, while EC1 to EC10 are transgenic positive. Arrows above the sequencing chromatograms indicate the location of indels in the target sequence, resulting in the overlap of peaks in the chromatograms. (b) Decoding of the mono and biallelic indels in *gfp* transgene of the *Setaria viridis* co-transforming EC1 to EC10 events compared with ECWT1 and ECWT2 plants (negative controls for edition, *gfp* gene wild-type). (c) Relative expression of *gfp* mRNA by qRT-PCR from EC1 to EC10 co-transforming events (T0 lines), ECWT1 and ECWT2 plants (GFP-positive control), and SvA10.1 wild-type plants (non-transgenic; negative control). Statistical difference between each independent event was confirmed using Tukey's HSD test (*p*-value<0.01).

according to *in silico* predictions (Supplementary Table S1). Sanger sequencing of PCR fragments amplified from these genomic regions showed no mutations in each region, demonstrating the specificity of gRNA targeting to the *gfp* gene sequence within transgene (Supplementary Figure S1B).

Discussion

The development of genome editing technology over the last 20 years has employed several types of nucleases (Boch et al. 2009; Christian et al. 2010; Durai et al. 2005; Joung and Sander 2013; Kim et al. 1996; Paques and Duchateau 2007; Smith et al. 2006; Streubel et al. 2012), but CRISPR/Cas9 has revolutionized genome editing because of its simplicity, versatility, efficiency, and specificity (Gil-Humanes et al. 2017; Li et al. 2016; Liang et al. 2017; Shan et al. 2014; Yin et al. 2017; Zhang et al. 2016). CRISPR/Cas9 has been used successfully in numerous organisms, including several plant species (Chang et al. 2016; Gao et al. 2015; Gerasimova et al. 2017; Jacobs et al. 2015; Jiang et al. 2013, 2014; Li et al. 2016; Lowder et al. 2015; Michno et al. 2015; Yin et al. 2015; Zhang et al. 2016). However, C4 grasses like maize, sorghum, *Brachiaria*, switchgrass, and sugarcane have highly complex genomes, which contributes to low efficiency for genome editing (Mohan 2016). Despite the economic importance of C4 crop plants as food crops or biomass for biofuel production, there are still few studies reporting successful genome editing by CRISPR/Cas9 in these plant species (Gerasimova et al. 2017). Also, genetic manipulation of these crops is laborious and time-consuming, as long periods are usually necessary to advance generations, making functional genomic studies difficult (Basso et al. 2019; Basso et al. 2020). The use of model plants is a common alternative for functional genomics studies, as work in these translates to the target C4 crop species (Nguyen et al. 2020; Santos et al. 2020). In this context, *S. viridis* serves as a model plant for important phylogenetically related C4 crops (Brutnell et al. 2015). Therefore, new resources and investigative tool development for *S. viridis* represent important advances. Huang et al. (2019) showed that CRISPR/Cas9 efficiently induced mutations in *S. viridis* to create new mutant alleles.

Here we describe a successful pipeline for *S. viridis* genome editing. We engineered a CRISPR/Cas9 binary vector to promote NHEJ. This vector coupled with a high-efficiency transformation protocol (Martins et al. 2015) generated ten independent gene editing events, all containing *indels* in the *gfp* target gene, in which 60% were biallelic. The efficiency achieved with this system was higher than reported with other approaches like transient expression of CRISPR/Cas9 components (Zhang et al. 2016), viral vectors (Yin et al. 2015), or

ribonucleoprotein complexes (Liang et al. 2017; Woo et al. 2015). Also, the efficiency was similar to or higher than DNA-integration-based genome-editing methods used in other monocots (Svitashev et al. 2016). Further, the pipeline describe here was simple, accurate, and highly efficient with *indels* equivalent to the CRISPR/Cas9_Trex2 system (Weiss et al. 2020). A contributor to the efficiency of editing observed here may be the use of strong constitutive promoters driving *Cas*9 nuclease and the gRNA, which improves the frequency of DSB due to high expression in transgenic cells at the early stages of dedifferentiation and differentiation (Gil-Humanes et al. 2017; Yang et al. 2017).

No *indels* occurred in potential off-target sites in the T0 events obtained here, confirming the specificity of the Cas9-gRNA combination, which is in part due to the low level of off-target cleavage when gRNAs are designed to be specific to target sequences (Young et al. 2019). The occurrence of *indels* in off-target sites and toxicity of strong and prolonged Cas9-gRNA expression in plant and bacteria are major concerns (Johnson et al. 2015; Lowder et al. 2015, 2016; Peng et al. 2016). However, highly target-specific gRNAs might eliminate the need for strong Cas9-gRNA expression (Osakabe et al. 2016). In addition, long term Cas9 cytotoxic effects have not been observed in most plant species. The use of the CRISPR/Cas9_Trex2 system to enhance *indels* in human cells indicated that Trex2 exonuclease increases frequency of off-target mutations (Chari et al. 2015). Weiss et al. (2020) demonstrated that CRISPR/Cas9_Trex2 system improves *indels* in *S. viridis* compared to the canonical CRISPR/Cas9 system, along with enhanced off-target mutations.

The results here showed codon optimized Cas9 nuclease was not cytotoxic for *E. coli*, *A. tumefaciens*, and *S. viridis* cells. Furthermore, *indels* introduced in *S. viridis* by this CRISPR/Cas9 system at single or multiple sites were stably inherited from the T0 to T1 generations, indicating Mendelian inheritance similar to that observed in other plant species (Yang et al. 2017; Zhang et al. 2016; Zhao et al. 2016) or even in *S. viridis* (Weiss et al. 2020). Transgene-free mutant plants can be obtained by self-crossing or backcrossing in the T1 generation from events carrying either homozygous or heterozygous for the CRISPR/Cas9 T-DNA (Zhang et al. 2014). Recently, transgene-free plants were obtained in the T0 generation with new strategies based on transient expression of CRISPR/Cas9 DNA or RNA (Zhang et al. 2016), viral vectors (Gil-Humanes et al. 2017; Yin et al. 2015), and ribonucleoprotein complexes (Liang et al. 2017; Svitashev et al. 2016; Woo et al. 2015).

In summary, we developed an optimized CRISPR/Cas9 binary vector, and this study confirms the efficiency of a pipeline for site-directed editing using this CRISPR/Cas9 system in *S. viridis*, similar to that shown by

Weiss et al. (2020). This work shows that promoting NHEJ pathway activity leads to accurate gene editing in *S. viridis*, with 60% of the transgenic events in a biallelic state at the T0 generation. This demonstrates the CRISPR/Cas9 NHEJ system is highly efficient in *S. viridis*. Furthermore, our results showed that a binary vector containing Cas9 nuclease codon-optimized for monocots and flanked by two NLS driven by strong constitutive promoters, and an optimized gRNA sequence driven by specific promoters were highly efficient in generating site-specific and biallelic *indels* in the *S. viridis* genome. Currently, our group is using this pipeline for genome editing in the sugarcane genome and we expect it to be effective in other important C4 grass and monocot crops.

Conclusion

In this work, we have successfully established a pipeline using the CRISPR/Cas9 NHEJ system for efficient editing and knock-out of target genes in *S. viridis*, which utilizes a new CRISPR/Cas9 binary vector, which exploits the NHEJ pathway, that is optimized and engineered for C4 monocots. This pipeline is expected to be useful for reverse genetics studies or to introduce desirable agronomic traits into economically important C4 crops such as maize, Brachiaria, wheat, rice, sorghum, and sugarcane.

Authors' contributions

MFB, TRS, and HBCM planned and designed CRISPR/Cas9 NHEJ strategy for *gfp* transgene editing and knockout in *S. viridis* accession A10.1; KED and BDBC carried out the *S. viridis gfp* transformation; MFB carried out the co-transformation of *S. viridis* using the CRISPR/Cas9 NHEJ binary vector, conducted biological and molecular analyses, wrote the manuscript, and prepared the figures and tables; WRS, BOG, and AKK provided inputs in the manuscript. All authors revised and approved the final version of the manuscript.

Competing interests

All authors declare that the research was carried out in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for publication or Ethics approval and consent to participate

Not applicable.

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