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Short communication

Systematic identification of endogenous RNA polymerase III promoters for efficient RNA guide-based genome editing technologies in maize

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ABSTRACT

Single-guide RNA (sgRNA) is one of the two core components of the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) genome-editing technology. We established an *in vitro* Traffic Light Reporter (TLR) system, which is designated as the same colors as traffic lights such as green, red and yellow were produced in cells. The TLR can be readily used in maize mesophyll protoplast for a quick test of promoter activity. The TLR assay indicates the variation in transcription activities of the seven Pol III promoters, from 3.4% (U6-1) to over 21.0% (U6-6). The U6-2 promoter, which was constructed to drive sgRNA expression targeting the *ZmWx1* gene, yielded mutation efficiencies ranging from 48.5% to 97.1%. Based on the reported and unpublished data, the *in vitro* TLR assay results were confirmed to be a readily system and may be extended to other plant species amenable to efficient genome editing *via* CRISPR/Cas. Our efforts provide an efficient method of identifying native Pol III-recognized promoters for RNA guide-based genome-editing systems in maize.

1. Introduction

RNA-guided engineered editing effectors derived from the bacteria adaptive immune system, designated as CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) nucleases [1,2], RNA effectors [3,4], and modification enzymes [5,6], enable versatile genome editing and represent exciting tools for application in diverse organisms. All of these systems depend essentially upon efficient nuclear-localized transcription of single-guide RNA

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(sgRNA) driven by RNA polymerase III (Pol III) promoters [1]. However, sgRNA expression is the limiting factor in the optimization of targeting and mutagenesis [7]. Recently, an alternative method to produce sgRNA using the Pol II promoter coupled with self-processing ribozyme-flanked RNAs was developed [8]. However, high and stable small nuclear RNA transcriptional activity of RNA Pol III, which occupies approximately 40% of total RNA [9], suggests that the Pol III promoter might play a primary role in RNA-guided genome editing strategies. Spliceosomal RNAs, such as U6, are conserved from yeast to mammals [10]. However, it is rare that foreign U6 or U3 (Pol III) promoters work well in RNA-guided genome-editing technologies applied across species [11]. For this reason, the identification of endogenous RNA Pol III promoters is one of the fundamental steps in optimizing RNA-guided genome-editing systems in target organisms. The objective of this study was to perform a systematic evaluation of endogenous Pol III promoters for RNA-guided genome technologies in maize, one of the most important cereal crops in the world.

2. Materials and methods

2.1. DNA extraction and isolation of ZmU6 promoters and ZmU3 promoter

DNA was isolated and purified using the DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's protocols. ZmU6 promoters and the ZmU3 promoter were amplified from the maize inbred 'Zheng 58'. PCR was performed to amplify the promoters using KOD-plus polymerase (Catalog#: KOD-401, TOYOBO Life Science Department, Osaka, Japan) with specific primers (Table S1). The PCR amplicons were cloned into the pEASY-blunt vector (Transgene, Beijing, China) and then sequenced following standard protocols.

2.2. Construction of the Traffic Light Reporter (TLR) system vector

The TLR system vector has an artificial target 23 bp DNA sequence, 5′-GAGAGAGCGTGTGTCGTCTCCGG G-3′, between the start codon, ATG, and an *eGFP* followed by a *DsRed-2* reading frame and the two gene (*eGFP* and *DsRed-2*) were separated by two nucleotides, CC.

The maize U6 and U3 promoters were used to drive the sgRNA gene, and the promoters and sgRNA genes were cloned into the TLR vector following the manufacturer's suggested protocols. For the U3 promoter, the first base was dropped from the target sequence so that transcription would start at an "A". Thus, the target sequence for the maize U3 promoter was 5′-AGAGAGCGTGTGTCGTCTCCGGG-3′, in which the last G is in the *eGFP* open reading frame.

2.3. Mesophyll protoplast culture and transformation

Protoplasts were prepared from the seedling leaves of the maize hybrid Zhongdan 99, based on a previously reported method [12]. A 200-μL volume of mesophyll protoplast containing 4×10^5 protoplasts with 40 μg plasmid DNA (20 μg of the TLR and 20 μg of the Ubi promoter drived the *sp*CRISPR-Cas9 gene vector) was mixed with 220 mL of 45% (4.5 g per 10 mL, [w/v]) polyethylene glycol PEG-4000 (Sigma-Fluka, catalog number 81242, USA) solution at room temperature in the dark for 15 min. After addition of 880 mL W5 solution (154 mmol L^{-1} NaCl, 125 mmol L^{-1} $CaCl_2$, 5 mmol L^{-1} KCl, 5 mmol L^{-1} glucose, 0.03% MES, pH to 5.8 with KOH.) to stop the reaction, the protoplasts were harvested by centrifugation at 100*g* for 3 min. They were resuspended in 2 mL W5 solution and incubated in 6-well plates in the dark at 28 °C for 48 h.

2.4. Confocal laser scanning microscopy and flow cytometry

eGFP and DsRed-2 signals resulting from induced mutations were detected with a Zeiss (LSM700, Germany) microscope using 488-nm (eGFP) and 558-nm (DsRed-2) excitation wavelengths with 10 times eye lens, 20 or 40 times objective lens magnification. Flow cytometry of TLR samples was performed on a BD FACSAriaIII instrument (BD, USA) using 488-nm (eGFP, FITC) and 558-nm (DsRed-2) excitation wavelengths. Briefly, protoplasts were harvested after incubation by centrifugation at 100*g* for 3 min and resuspended in 200 μL W5 solution. The suspension was loaded into a Round-Bottom Tubes (BD, USA). Gates were set to capture positive cells, using a negative control to determine autofluorescence thresholds for non-expressing cells.

2.5. Construction of the RNA-guided Cas9 vector

The expression cassette of the maize ubiquitin promoter was used to derive the modified coding sequence of *SpCas9* gene, which was constructed based on the CPB vector. The nuclear localization signal sequence of SV40 and nucleoplasmin were embedded at either end of the expressed Cas9 protein. The guide RNA sequence 5′-CCGACTACCCGGAGCTGAACCTC-3′ was selected to target the maize *ZmWx1* gene within the coding region of exon 7 at chromosome 9 from 23,267,684 to 23,271,612 bp (AGP v3.0). The underlined CCG represents the proto-adjacent-motif (PAM). The maize U6-2 promoter described in this report was used to express the sgRNA gene targeting *ZmWx1*.

2.6. Maize transformation

ZC01, a private inbred line, was transformed based on a modified *Agrobacterium tumefaciens* (EHA105 strain)-mediated immature embryo transformation protocol [13]. The procedure and the characterization of transformation products were essentially as previously described [14].

2.7. Detection of genome modifications

Genomic DNA was extracted from maize T0 and T1 *Wx1* mutants. PCR was performed to amplify the genomic regions surrounding the CRISPR target sites using KOD-plus polymerase (TOYOBO, Life Science Department, Osaka, Japan). The PCR amplicons were cloned into the pEASY-blunt vector (Transgen) and sequenced. M13 primers were used for Sanger sequencing on an ABI3730 instrument (Applied Biosystems, California, USA).

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