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Targeted Mutagenesis in *Zea mays* Using TALENs and the CRISPR/Cas System

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ABSTRACT

Transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems have emerged as powerful tools for genome editing in a variety of species. Here, we report, for the first time, targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. We designed five TALENs targeting 4 genes, namely *ZmPDS*, *ZmIPK1A*, *ZmIPK*, *ZmMRP4*, and obtained targeting efficiencies of up to 23.1% in protoplasts, and about 13.3% to 39.1% of the transgenic plants were somatic mutations. Also, we constructed two gRNAs targeting the *ZmIPK* gene in maize protoplasts, at frequencies of 16.4% and 19.1%, respectively. In addition, the CRISPR/Cas system induced targeted mutations in *Z. mays* protoplasts with efficiencies (13.1%) similar to those obtained with TALENs (9.1%). Our results show that both TALENs and the CRISPR/Cas system can be used for genome modification in maize.

KEYWORDS: TAL-effector nucleases; CRISPR/Cas system; Knock-out; *Zea mays*

INTRODUCTION

The development of whole genome sequencing and genomics has underlined the need for powerful genome editing tools to elucidate gene functions. In the past few years, artificial designed nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems have been widely used for targeted genome modification in plant species such as *Arabidopsis* (Zhang et al., 2010; Li et al., 2013; Qi et al., 2013), tobacco (Nekrasov et al., 2013; Zhang et al., 2013), rice (Li et al., 2012; Shan et al., 2013a, 2013b), barley (Wendt et al., 2013), soybean (Curtin et al., 2011), *Brachypodium* (Shan et al., 2013b) and maize (Shukla et al., 2009). All these nucleases consist of DNA binding domains together with non-specific nuclease domains that generate double-strand breaks (DSBs). The DSBs are mainly repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR) pathway (Chen et al., 2013; Gaj et al., 2013). NHEJ simply rejoins the broken DNA ends in an error-prone fashion and often results in small deletions or insertions. In the HR pathway, DSBs are correctly repaired using a homologous donor DNA as template.

TALENs and CRISPR/Cas systems have tremendous advantages over ZFNs, because of their one-to-one recognition of nucleotides, which makes them easier to design and construct (Boch et al., 2009; Cong et al., 2013; Mali et al., 2013). TALENs consist of customizable TALE DNA binding domains fused with non-specific *Fok* I cleavage domains. TALEs comprise a series of 34-amino-acid repeats, each with a repeat-variable di-residue (RVD) at positions 12 and 13 that can be used for recognizing a single target nucleotide (Fig. 1A). This modular architecture has been successfully produced by many different methods and makes TALENs an

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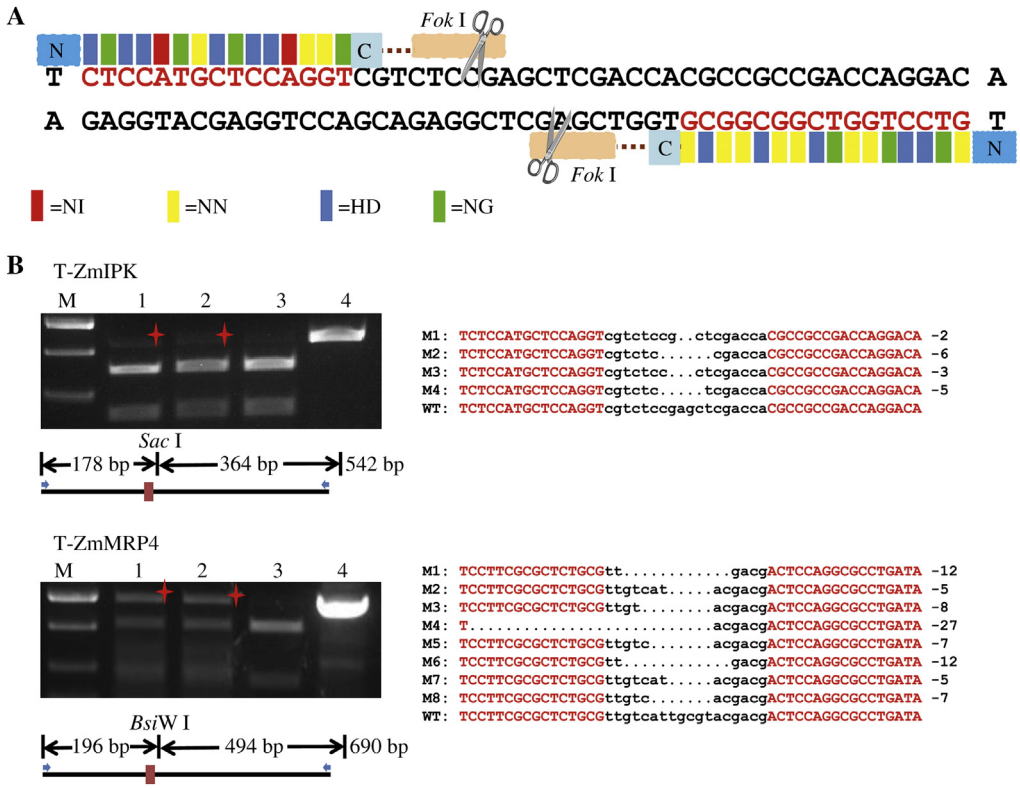

Fig. 1. Targeted mutagenesis using TALENs in maize protoplasts.
A: Schematic representation of a TALEN binding to its target DNA (*ZmIPK*). The left and right binding sites are highlighted in red. The colored boxes represent the TALE repeats. Each recognizes one nucleotide and determines the sequence specificity. *Fok* I endonuclease (orange) is dimerized to cleave the target sequence. **B:** PCR/Restriction enzyme assays were performed to detect mutations at the *ZmIPK* and *ZmMRP4* loci (left panel). Lanes 1 and 2 represent PCR products of samples treated with the respective TALENs. Lanes 3 and 4 indicate digested and undigested wild-type controls, respectively. Representative sequences of the mutations induced by TALENs are shown in the right panel. The spacer and deletions are indicated by lower-case letters and black dots, respectively. The net change in length is shown to the right of each sequence (−, deletion).

efficient procedure for genome editing (Cermak et al., 2011; Schmid-Burgk et al., 2012; Wang et al., 2012). The recently discovered type II CRISPR/Cas system requires a dual-tracrRNA:crRNA (gRNA) to guide the non-specific nuclease, Cas9, for DNA cleavage. The RuvC and HNH nuclease domains in Cas9 are responsible for cleaving the complementary DNA strands (Fig. 3A) (Gasiunas et al., 2012). The gRNA recognizes any genomic locus that is followed by a 5′-NGG protospacer-adjacent motif (PAM), and a 20-nt sequence preceding the PAM directs the Cas9 to cleave the target sequence by Watson-Crick base pairing. The simplicity of the cloning strategy and the fewer limitations of potential target sites make the CRISPR/Cas system very appealing.

Maize (*Zea mays*) is an important model organism for fundamental research into the inheritance and functions of genes, and an important crop, yielding 12 billion bushels of grain in the USA alone in 2008. Phytic acid (PA), inositol 1,2,3,4,5,6-hexakisphosphate, is a natural product present in maize seeds, which represents about 75% of the total seed phosphorus. However, PA is an anti-nutritional compound, as it cannot be digested by monogastric animals and can cause environment pollution. Thus, it is important to reduce the PA content of maize seeds. We describe here the design and construction of TALENs and a gRNA:Cas9 construct that target the *ZmIPK1A* (Sun et al., 2007), *ZmIPK* (Shi et al., 2003), and *ZmMRP4* (Shi et al., 2007) genes encoding enzymes that catalyze three steps in the PA biosynthetic pathway. We demonstrate that both TALEN and CRISPR systems can introduce mutations at the target sites in transgenic maize. To our knowledge, this is the first study to demonstrate targeted mutagenesis in maize using both TALENs and CRISPR/Cas systems. We anticipate that TALEN and CRISPR technologies will make targeted gene modification a routine practice in this economically important crop and substantially increase the potential for molecular breeding.

RESULTS AND DISCUSSION

Because plant tissue culture and transformation is time-consuming, it is necessary to test the activity of TALENs using a transient expression system before using them for genetic transformation. In this study, we carried out protoplast transformation based on previous work (Shan et al., 2013b) with slight modifications. Five pairs of TALENs (T-ZmPDS-1, T-ZmPDS-2, T-ZmIPK1A, T-ZmIPK and T-ZmMRP4) were generated targeting four endogenous loci, namely *ZmPDS* (NM_001111911), *ZmIPK1A* (EF447274), *ZmIPK* (AY172635), *ZmMRP4* (EF586878), and each target site contained a unique restriction