Available online at www.sciencedirect.com

ScienceDirect

Journal of Genetics and Genomics 41 (2014) 63–68

JGG

ORIGINAL RESEARCH

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

Zhen Liang, Kang Zhang, Kunling Chen, Caixia Gao*

State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Received 12 November 2013; revised 5 December 2013; accepted 5 December 2013
Available online 14 December 2013

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

* Corresponding author. Tel/fax: +86 10 6480 7727.
E-mail address: cxgao@genetics.ac.cn (C. Gao).

http://dx.doi.org/10.1016/j.jgg.2013.12.001

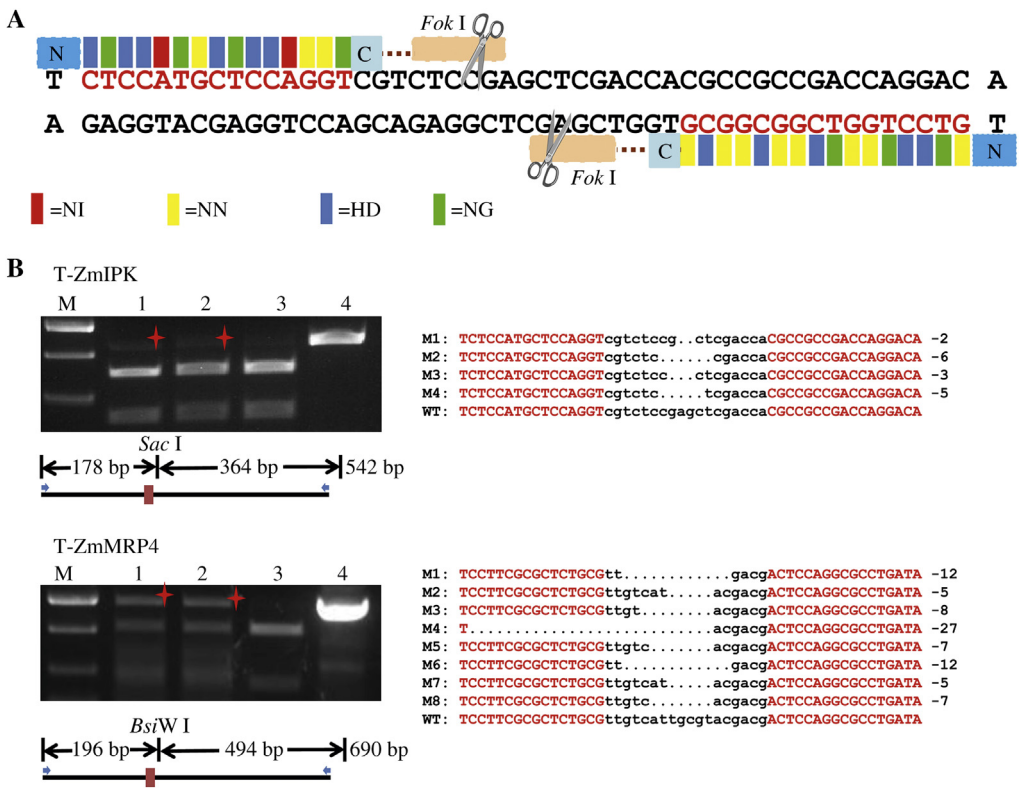

Fig. 1. Targeted mutagenesis using TALENs in maize protoplasts.
A: Schematic representation of a TALEN binding to its target DNA (*ZmIPK*). The left and right binding sites are highlighted in red. The colored boxes represent the TALE repeats. Each recognizes one nucleotide and determines the sequence 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

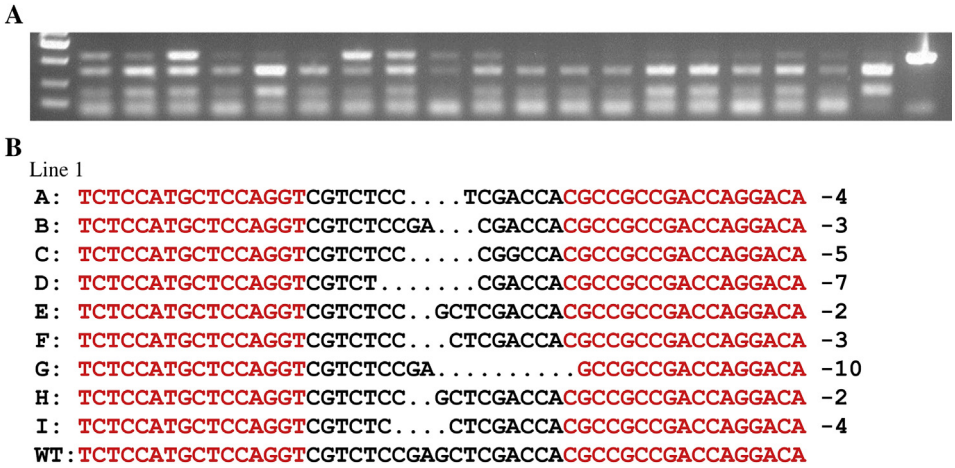

Fig. 2. TALEN-induced *ZmIPK* mutations in transgenic plants.

A: A representative gel of the PCR products from independent transgenic seedlings. **B:** Sequence alignment of the WT (bottom) sequence and several independent amplicons from one plant. The TALE binding sites are indicated in red. The deletions are shown as black dots, and the net change in length is shown to the right of each sequence (−, deletion).

enzyme site within the spacer (Table S1). As the restriction enzyme sites were in the middle of the spacer, this approach roughly resembles mutagenesis caused by NHEJ repair. All of the TALEN repeat arrays were assembled by the Golden Gate assembly method and cloned into expression vector pZHY501 under the control of the 35S promoter (Zhang et al., 2013).

Each pair of TALEN-encoding constructs was introduced into maize protoplasts by PEG-mediated transformation. After 40–48 h cultivation, genomic DNA was isolated from the protoplasts and fragments encompassing the target sites were amplified by PCR. These were then digested with the corresponding restriction enzymes, visualized by agarose gel electrophoresis, and full length PCR products were then cloned and sequenced. A group of indels, with 2–17 bp deletions are shown in Fig. 1B. Efficiencies for T-ZmIPK and T-ZmMRP4 were 9.1% and 23.1%, respectively, as estimated from bands intensities. For T-ZmPDS-1, T-ZmPDS-2, and T-ZmIPK1A, enrichment PCR was carried out to detect mutations. The genomic DNAs were digested with the corresponding restriction enzymes (Table S1) and PCR was performed with primers flanking the target sites. After further digestion with the same restriction enzymes, undigested sequences in the samples incubated with TALENs carried mutations (Fig. S1).

To evaluate whether TALENs can induce mutations in plants, all five pairs of TALENs were introduced into Hi-II plants by *Agrobacterium*-mediated transformation. We carried out enrichment PCR to detect mutations induced by T-ZmIPK (Fig. 2A). We purified the undigested PCR products from individual plants and cloned them into pEasy-TI vector (Transgen, China). Ten clones were picked at random for sequence analysis. The results showed that different mutation patterns were present at the target sites (Fig. 2B). The mutations in other lines are shown in Fig. S2. With T-ZmIPK, about 39.1% of the independent plants generated mutations in *ZmIPK*, and similar results were obtained with T-ZmIPK1A, T-ZmPDS-1 and T-ZmPDS-2. The mutant sequences are presented in Fig. S2. Similar results have been obtained in barley (Wendt et al., 2013). The presence of somatic mutagenesis of T_0 plants may result from a delayed and sustained cleavage activity due to the TALENs. Different mutations may be transmitted to T_1 plants by self-fertilization, so that T_1 plants derived from a single plant can carry different mutations. Studies in *Xenopus* embryos have shown that mosaic mutations in G_0 are transmitted through the germ line (Lei et al., 2013). In summary, our data indicate that TALENs are able to bind to target sites and induce somatic mutations in *Z. mays*.

Recent advances in understanding of CRISPR/Cas system provide an easier approach to targeted mutations in eukaryotes (Cong et al., 2013; Mali et al., 2013). To optimize the CRISPR/Cas system in maize, we used the maize U3 promoter to drive gRNA expression. The codon-optimized Cas9 and gRNA scaffold have been described previously (Shan et al., 2013a).

To see whether the CRISPR/Cas system induces mutations at target sites, we designed two gRNAs targeting the *ZmIPK* (AY172635) gene, preceded by an NGG, the protospacer-adjacent motif (PAM). For ease of analysis, each gRNA had a restriction enzyme target site at the Cas9 cleavage site (*Sac* I for gRNA-1, *Bam*H I for gRNA-2), 3 bp before the PAM motif. We used the same strategy as above to detect mutations. Sequence analysis revealed 1–44 bp deletions and a 125 bp insertion (Fig. 3B), confirming the presence of mutations at the target sites. PCR/RE (PCR/Restriction enzyme assay) assays were performed to measure the mutation efficiency. The efficiencies at gRNA-1 and gRNA-2 were 16.4% and 19.1% (Fig. 3B), respectively. We also compared for the frequencies mutations induced by T-ZmIPK and gRNA-1, targeting the same site. As shown in Fig. 3C, CRISPR/Cas (13.1%) is capable of inducing mutations efficiently as TALENs (9.1%).

In this study, we have demonstrated that TALENs and the CRISPR/Cas system are able to induce targeted mutagenesis

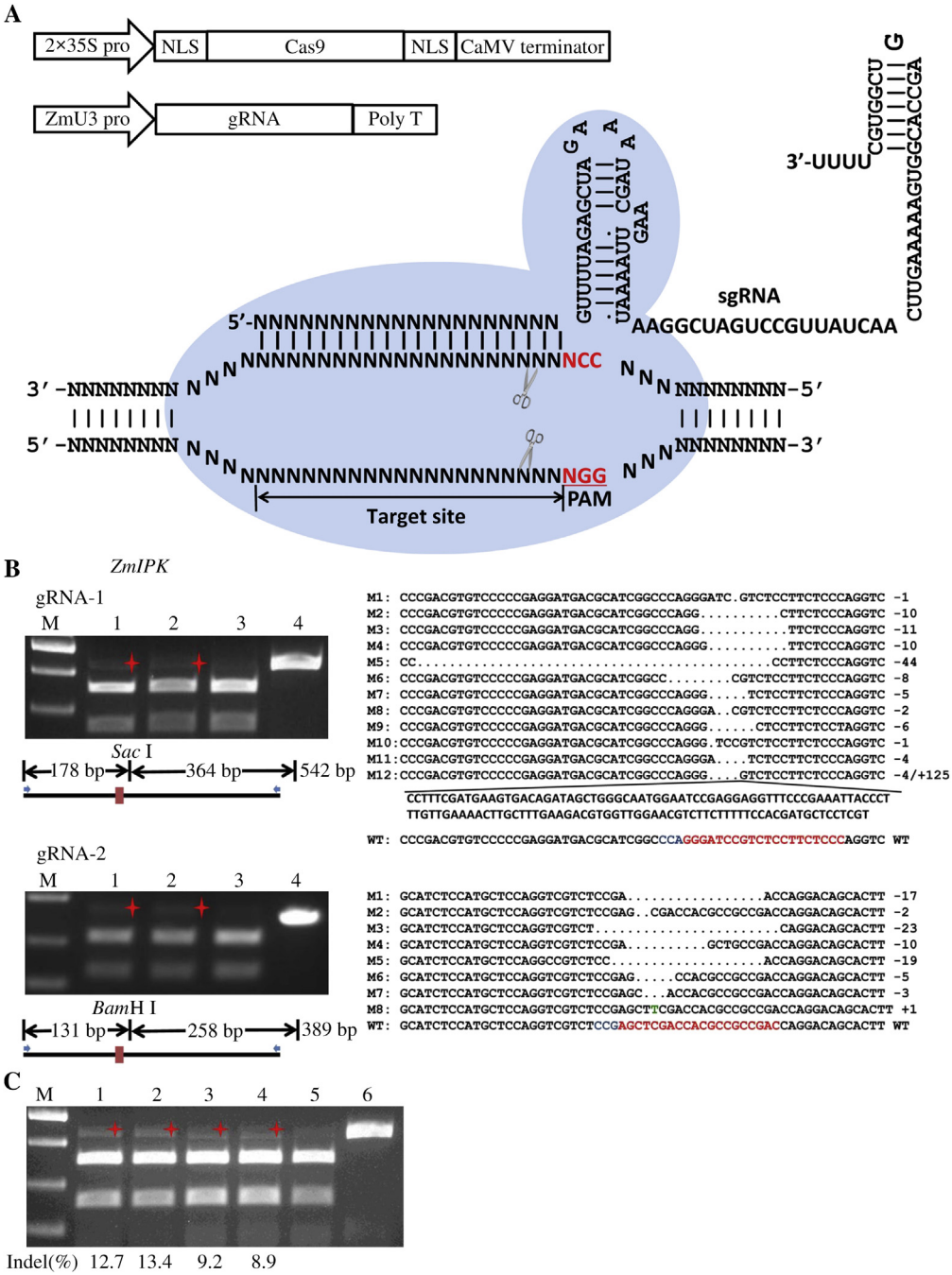

Fig. 3. Targeted mutagenesis *via* the CRISPR/Cas system in maize protoplasts.

A: Schematic representation of the CRISPR/Cas system, including Cas9 and the gRNA. Cas9 flanked by two NLS signals is driven by two 35S promoters as described previously, and the gRNA is driven by the maize U3 promoter. PAM, the protospacer-adjacent motif, is highlighted in red. **B:** PCR/RE assays were performed to detect mutations (left panel). Lanes 1 and 2 represent PCR products of samples treated with the respective gRNA:Cas9. Lanes 3 and 4 represent digested and undigested wild-type controls, respectively. Representative sequences of the mutations induced by the CRISPR/Cas system are shown in the right panel. The wild-type sequence is given at the bottom, with the target sites highlighted in red and the PAM highlighted in blue. The deletions are shown as black dots. The net change in length is shown to the right of each sequence (+, insertion; −, deletion). **C:** Comparison of the mutation efficiencies of TALEN and CRISPR/Cas system at the *ZmIPK* locus. Lanes 1 and 2 represent samples treated with Cas9 and gRNA-1. Lanes 3 and 4 indicate samples treated with T-ZmIPK. Lanes 5 and 6 represent the digested and undigested PCR products of the wild-type control, respectively. Indels (%) indicate mutagenesis frequencies of small insertions and deletions.

in *Z. mays* with high and comparable efficiencies. Although TALENs are effective tools for genome editing, there are some limitations regarding the potential target sites, such as the need for T at position −1 (Doyle et al., 2012) and the fact that some TALENs fail to cause mutations. The recently developed CRISPR/Cas system seems to provide a complementary approach to TALENs, as it only requires the PAM (NGG) motif preceding the recognition sequence. In addition, the

CRISPR/Cas system has great advantages in terms of easy cloning and multiplex genome editing. However, the high-frequency of off-target mutations reported in human cells (not assessed in the present work) may seriously limit application of the CRISPR/Cas system (Fu et al., 2013; Pattanayak et al., 2013). Here, we conclude that both TALENs and CRISPR/Cas system can facilitate genome modification and functional genomic studies in maize.

MATERIALS AND METHODS

Construction of TALENs

The TALEN target sites used in this study were predicted by TAL Effector-Nucleotide Targeter 2.0 software (https://tale-nt.cac.cornell.edu/) (Doyle et al., 2012). The TALEN repeats were assembled using the Golden Gate strategy. TALEN Left/Right arrays were released by digestion with *Xba* I/*Bam*H I, and successively cloned into the intermediate vector pZHY013 as *Xba* I/*Bam*H I fragment and at *Nhe* I/*Bgl* II sites (Zhang et al., 2013). The entry vector was cloned into the destination vector, pGW6, which contains the *bar* gene as selectable marker and two 35S promoters to drive TALEN expression.

Cas9 and gRNA design

The Cas9 gene sequence used in this paper has been previously described (Shan et al., 2013a). The maize U3 promoter and the gRNA scaffold were amplified and cloned into pEasy Blunt vector (Transgen, China). The sequence of ZmU3-gRNA and the primers used are shown in Fig. S3 and Table S2. The synthesized target sequences were annealed and inserted into *Bbs* I-digested pU3-gRNA vector.

Maize protoplast transformation

Maize Hi-II seedlings were soaked in water overnight, and the seeds were transferred to Petri dishes between blotting papers for 2 days to germinate. The germinated seeds were then moved to the dark for 10–11 days at 24°C. Etiolated leaves were used in this study.

To obtain mesophyll protoplasts, the middle parts of second leaves were cut into strips and digested with enzyme solution (1.5% Cellulase R10, 0.3% Macerozyme R10, 0.6 mol/L mannitol, 10 mmol/L MES at pH 5.7, 1 mmol/L $CaCl_2$ and 0.1% BSA). A vacuum of −15 ∼ −20 (in Hg) was applied for 30 min, and the digestion was continued for 4–6 h with gentle shaking (90 r/min). The protoplasts were collected by filtration through a 40 μm nylon mesh and spun at 150 *g* for 3 min. After washing in W5 solution (154 mmol/L NaCl, 125 mmol/L $CaCl_2$, 5 mmol/L KCl and 4 mmol/L MES at pH 5.7), the protoplasts were resuspended in MMg solution (0.4 mol/L mannitol, 15 mmol/L $MgCl_2$ and 4 mmol/L MES at pH 5.7).

The protoplast transformation procedure was modified from an *Arabidopsis thaliana* transformation protocol. Twenty μg of plasmid DNA (10 μg plasmid for each if for co-transformation) was incubated with 200 μL protoplasts in 220 μL of PEG solution at room temperature in the dark for 15 min. After adding 880 μL W5 solution to stop the reaction, the protoplasts were harvested by centrifugation at 100 *g* for 3 min. They were resuspended in 2 mL WI solution (0.5 mol/L mannitol, 4 mmol/L KCl and 4 mmol/L MES at pH 5.7) and incubated in 6-well plates in the dark at 22°C for 48 h.

Detecting mutations by PCR/RE assays and Sanger sequencing

Genomic DNA was extracted using the DNA Quick Plant System (Tiangen, China). To detect the efficiencies of T-ZmIPK, T-ZmMRP4, gRNA-1 and gRNA-2 in protoplasts, PCR/RE assay were carried out. We performed PCR with genomic DNA using primers flanking the target sites, then digested the PCR products with the appropriate restriction enzymes (Table S1) and visualized the products by agarose gel electrophoresis. The mutation frequencies were calculated by measuring band intensities with UVP Vision Works LS Image Acquisition Analysis Software 7.0. To detect the activities of T-ZmPDS-1, T-ZmPDS-2 and T-ZmIPK1A, we used enrichment PCR to reduce the amount of unaltered wild-type DNA in the samples. The genomic DNA was pre-digested at 37°C for 1 h (300–500 ng DNA, 1× Fastdigest buffer, 1 μL restriction enzyme), and PCR was performed on the pre-digested DNA. The amplified PCR products were digested with restriction enzymes at 37°C for 3 h, and after separation on 1.2% agarose gels, undigested bands were gel purified and cloned into pEasy-TI vector for Sanger sequencing.

Agrobacterium transformation

Maize Hi-II plants were grown in the field at the experimental station of Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, in Sanya (Hainan Province, China) under normal field conditions. Immature embryos isolated 12–16 days after pollination were used for *Agrobacterium*-mediated transformation following the procedure of Ishida et al. (2007). Hygromycin-resistant plantlets regenerated after 9–12 weeks of selection, and about 30 to 50 transgenic lines were sampled from each transformation.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant Nos. 31271795 and 31200273).

SUPPLEMENTARY DATA

Fig. S1. Targeted indel mutations induced by TALENs in maize protoplasts.

Fig. S2. Sequences of TALEN-induced mutations in plants.

Fig. S3. Full DNA sequence of the gRNA expression vector and pZmU3-gRNA.

Table S1. Target loci and sequences of the mutations generated in maize by TALENs and the CRISPR/Cas system.

Table S2. List of primers used in this study, and their applications.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2013.12.001.

REFERENCES

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., Bonas, U., 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326, 1509−1512.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., Voytas, D.F., 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 39, e82.

Chen, K., Gao, C., 2013. TALENs: customizable molecular DNA scissors for genome engineering of plants. J. Genet. Genomics 40, 271−279.

Cong, L., Ran, F.A., Cox, D., Lin, S.L., Barretto, R., Habib, N., Hsu, P.D., Wu, X.B., Jiang, W.Y., Marraffini, L.A., Zhang, F., 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819−823.

Curtin, S.J., Zhang, F., Sander, J.D., Haun, W.J., Starker, C., Baltes, N.J., Reyon, D., Dahlborg, E.J., Goodwin, M.J., Coffman, A.P., Dobbs, D., Joung, J.K., Voytas, D.F., Stupar, R.M., 2011. Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol. 156, 466−473.

Doyle, E.L., Booher, N.J., Standage, D.S., Voytas, D.F., Brendel, V.P., Vandyk, J.K., Bogdanove, A.J., 2012. TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res. 40, W117−W122.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., Sander, J.D., 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. 31, 822−826.

Gaj, T., Gersbach, C.A., Barbas 3rd, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 31, 397−405.

Gasiunas, G., Barrangou, R., Horvath, P., Siksnys, V., 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Natl. Acad. Sci. USA 109, E2579−E2586.

Ishida, Y., Hiei, Y., Komari, T., 2007. Agrobacterium-mediated transformation of maize. Nat. Protocols 2, 1614−1621.

Lei, Y., Guo, X., Deng, Y., Chen, Y., Zhao, H., 2013. Generation of gene disruptions by transcription activator-like effector nucleases (TALENs) in *Xenopus* tropicalis embryos. Cell Biosci. 3, 21.

Li, J.F., Norville, J., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., Sheen, J., 2013. Multiplex and homologous recombination−mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. Nat. Biotechnol. 31, 688−691.

Li, T., Liu, B., Spalding, M.H., Weeks, D.P., Yang, B., 2012. High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat. Biotechnol. 30, 390−392.

Mali, P., Yang, L.H., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013. RNA-guided human genome engineering *via* Cas9. Science 339, 823−826.

Nekrasov, V., S, B., Weigel, D., Jones, J.D.G., Kamoun, S., 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31, 691−693.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., Liu, D.R., 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat. Biotechnol. 31, 839−843.

Qi, Y., Li, X., Zhang, Y., Starker, C.G., Baltes, N.J., Zhang, F., Sander, J.D., Reyon, D., Joung, J.K., Voytas, D.F., 2013. Targeted deletion and inversion of tandemly arrayed genes in *Arabidopsis thaliana* using zinc finger nucleases. G3 (Bethesda) 3, 1707−1715.

Shan, Q., Wang, Y., Chen, K., Liang, Z., Li, J., Zhang, Y., Zhang, K., Liu, J., Voytas, D.F., Zheng, X., Zhang, Y., Gao, C., 2013a. Rapid and efficient gene modification in rice and *Brachypodium* using TALENs. Mol. Plant 6, 1365−1368.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J., Qiu, J., Gao, C., 2013b. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31, 686−688.

Schmid-Burgk, J.L., Schmidt, T., Kaiser, V., Honing, K., Hornung, V., 2012. A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. Nat. Biotechnol. 31, 76−81.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P., Glassman, K., 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nat. Biotechnol. 25, 930−937.

Shi, J., Wang, H., Wu, Y., Hazebroek, J., Meeley, R.B., Ertl, D.S., 2003. The maize low-phytic acid mutant *lpa2* is caused by mutation in an inositol phosphate kinase gene. Plant Physiol. 131, 507−515.

Shukla, V.K., Doyon, Y., Miller, J.C., DeKelver, R.C., Moehle, E.A., Worden, S.E., Mitchell, J.C., Arnold, N.L., Gopalan, S., Meng, X., Choi, V.M., Rock, J.M., Wu, Y.Y., Katibah, G.E., Zhifang, G., McCaskill, D., Simpson, M.A., Blakeslee, B., Greenwalt, S.A., Butler, H.J., Hinkley, S.J., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., 2009. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459, 437−441.

Sun, Y., Thompson, M., Lin, G., Butler, H., Gao, Z., Thornburgh, S., Yau, K., Smith, D.A., Shukla, V.K., 2007. Inositol 1,3,4,5,6-pentakisphosphate 2-kinase from maize: molecular and biochemical characterization. Plant Physiol. 144, 1278−1291.

Wang, Z., Li, J., Huang, H., Wang, G.C., Jiang, M.J., Yin, S.Y., Sun, C.H., Zhang, H.S., Zhuang, F.F., Xi, J.J., 2012. An integrated chip for the high-throughput synthesis of transcription activator-like effectors. Angew. Chem. Int. Ed. Engl. 51, 8505−8508.

Wendt, T., Holm, P.B., Starker, C.G., Christian, M., Voytas, D.F., Brinch-Pedersen, H., Holme, I.B., 2013. TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. Plant Mol. Biol. 83, 279−285.

Zhang, F., Maeder, M.L., Unger-Wallace, E., Hoshaw, J.P., Reyon, D., Christian, M., Li, X., Pierick, C.J., Dobbs, D., Peterson, T., Joung, J.K., Voytas, D.F., 2010. High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc. Natl. Acad. Sci. USA 107, 12028−12033.

Zhang, Y., Zhang, F., Li, X., Baller, J.A., Qi, Y., Starker, C.G., Bogdanove, A.J., Voytas, D.F., 2013. Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol. 161, 20−27.