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An efficient TALEN mutagenesis system in rice

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ABSTRACT

Targeted gene mutagenesis is a powerful tool for elucidating gene function and facilitating genetic improvement in rice. TALENs (transcription activator-like effector nucleases), consisting of a custom TALE DNA binding domain fused to a nonspecific *Fok*I cleavage domain, are one of the most efficient genome engineering methods developed to date. The technology of TALENs allows DNA double-strand breaks (DSBs) to be introduced into predetermined chromosomal loci. DSBs trigger DNA repair mechanisms and can result in loss of gene function by error-prone non-homologous end joining (NHEJ), or they can be exploited to modify gene function or activity by precise homologous recombination (HR). In this paper, we describe a detailed protocol for constructing TALEN expression vectors, assessing nuclease activities *in vivo* using rice protoplast-based assays, generating and introducing TALEN DNAs into embryogenic calluses of rice and identifying TALEN-generated mutations at targeted genomic sites. Using these methods, T_0 rice plants resulting from TALEN mutagenesis can be produced within 4–5 months.

1. Introduction

Rice (*Oryza sativa*) is one of the most important food crops, feeding nearly 50% of the world's population. It is also a much-studied model species of monocotyledon for gene function analysis. The completion and annotation of the rice genome, including both *indica* [1] and *japonica* [2] subspecies, provides a great deal of sequence information for large scale elucidation of gene function at the whole genome level; it also intensifies the need for methods of generating targeted mutations in specific genes. Current mutagenesis approaches, such as insertion of T-DNA [3,4] or transposable elements [5], chemical mutagenesis with ethyl methanesulfonate (EMS) for TILLING [6] and radiation mutagenesis [7] have been widely used and have yielded many mutants. However, these methods cannot target specific genes and require laborious works to identify the determined phenotype. Other reverse genetics approaches, such as RNAi [8], knock down the expression of specific genes; however, it is hard to achieve null phenotypes with them and the knock-downs cannot always be recovered in the progeny. Thus, reliable and efficient genome modification methods would be highly required to create targeted mutagenesis of interesting genes.

Transcription activator-like effector nucleases (TALENs) are one of the most powerful targeted genome modification technologies. TALENs are composed of an engineered specific DNA binding domain of a TALE fused to a nonspecific *Fok*I cleavage domain (Fig. 1A). Transcription activator-like effectors (TALEs), secreted by the plant pathogenic bacterial genus *Xanthomonas*, are major virulence factors that cause disease in plants such as rice by activating the transcription of specific target genes [9,10]. The central DNA binding domain (DBD) of a TALE contains tandem arrays of highly conserved 34 amino acid repeats [10]. The amino acid sequences of the repeats are nearly identical except for the di-residues at positions 12 and 13, designated "repeat variable di-residues" (RVDs). Each RVD specifically recognizes one nucleotide according to the following rules: HD recognizes cytosine (C), NG recognizes thymine (T), NI recognizes adenosine (A), and NN recognizes guanine (G) or adenosine (A) [11,12]. The simple nature of the TALE-DNA recognition code can be used to generate custom DBDs consisting of TALEs constructed to bind to the desired target DNA sequences. In this way TALENs have been developed as efficient tools for targeted genome modification [13–15]. Because FokI functions as a dimer, TALENs designed as pairs allow two monomers to bind at adjacent sites separated by a DNA spacer consisting of 15–30 nucleotides, and to cleave the target site [16]. Each TALEN is usually designed to bind to 14–20 nucleotides; thus the pair of TALENs can specifically recognize 50–60 nucleotides (including spacer sequences) which are enough to target a unique site in the genome [17]. DNA double strand breaks (DSBs) introduced by TALENs at predetermined sites in the genome activate DNA repair either by homologous recombination (HR) or error-prone non-homologous end joining (NHEJ) [18]. If DSBs are repaired by HR, which is activated by the homologous DNA template surrounding

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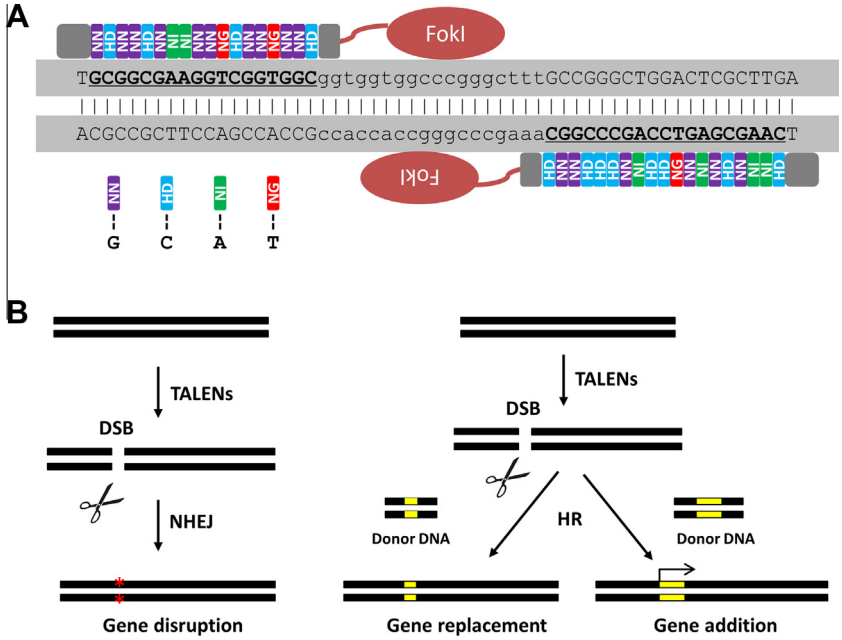

Fig. 1. Targeted mutagenesis via engineered TALENs. (A) Structure of a TALEN binding to its target gene (*OsBRII*). The colored boxes denote the TAL effector repeats. Each color represents a different repeat variable di-residue (RVD). FokI endonuclease is fused to the C-terminal domains. NI, HD, NN and NG recognize A, C, G and T, respectively. Target sites are in bold character and underlined; the spacer region is in lower case letters. (B) TALEN-induced double-strand breaks (DSBs) in a gene locus can be repaired by either non-homologous end-joining (NHEJ; left panel) or homologous recombination (HR; right panel). NHEJ-mediated repair leads to insertion or deletion (indel) mutations. HR with double-stranded donor DNA templates can lead to the introduction of precise nucleotide substitutions or insertions.

the DSBs, this can lead to gene addition or replacement (Fig. 1B). Alternatively, DSBs can be repaired by NHEJ, an error-prone pathway that often simply rejoins broken DNA imprecisely. NHEJ can be employed to create frame-shift knock-out mutations involving small deletions or insertions at the target sites (Fig. 1B). NHEJ is the dominant DNA repair pathway in higher plants [19]. Up to now, targeted mutagenesis in plants has depended mainly on the NHEJ pathway. In the past four years, TALEN-mediated targeted genome modification has been successfully adopted in yeast [20], nematode [21], fruit fly [22–24], human cells [25–27], silkworm [28], livestock [29], rat [30,31], plants [13,16,32–37], *Xenopus* embryos [38], zebrafish [39–44] and many other organisms.

Apart from TALENs, several other sequence-specific genome engineering tools, such as zinc finger nucleases (ZFNs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system, have been widely used. ZFNs were the first custom-designed nucleases engineered to cleave at specific DNA sequences in the late 1990s [45]. ZFNs bind DNA through an array of engineered zinc finger proteins, which are fused to the FokI cleavage domain. Although considerable achievements have been made with it [46], this tool remains difficult to use for many reasons, such as the difficulty in designing the constructs, the context dependence of the repeat units, frequent off-target effects and limited target sites [47]. Very recently, the RNA-based CRISPR/Cas system has been successfully used in a variety of organisms, including plants [48]. Unlike ZFNs and TALENs, CRISPR/Cas only needs to synthesize a single customized gRNA for each new target site, and the Cas9 protein does not requires reengineering [49–51]. The CRISPR system is therefore much more straightforward than ZFNs or TALENs. But the high off-target rates reported in human cells and other organisms suggest this method has to be optimized [52,53].

To date TALEN technology has been applied in plant species including *Arabidopsis* [13,16,35], tobacco [33,36], rice [34,37], *Brachypodium* [34], and barley [32]. The first successful TALEN-mediated genome editing experiments in plants was reported by the Voytas group, who assembled pairs of TALENs targeting the *Arabidopsis* gene *ADH1* and confirmed the action of the TALENs in a yeast-based SSA assay [13]. A year later, Cermak et al. in the same laboratory developed a Golden Gate method for efficiently assembling TALEN constructs with custom repeat arrays, and the same *Arabidopsis* gene *ADH1* was targeted in *Arabidopsis* protoplasts [16]. The first use of TALENs for trait improvement in rice was made by Li et al. [37]. They introduced a mutation in the promoter region of the sucrose–efflux transporter gene, *OsSWEET14*, which led to increased resistance to bacterial blight. Zhang et al. optimized the TALEN scaffold and carried out TALEN-mediated gene replacement in the *ALS* genes of tobacco protoplasts [33]. Recently, Wendt et al. [32] targeted a region that contains a group of regulatory motifs in the promoter of the barley phytase gene, *HvPAPhy*. Christian et al. found that TALEN-induced mutations in *Arabidopsis* were transmitted to the next generation at frequencies of 1.5–12% [35].

In this article we provide protocols for TALEN-mediated targeted genome mutagenesis in rice, including TALEN Golden Gate assembly and the construction of plant expression vectors, validation of TALEN activity in rice protoplast, TALEN transformation in rice and screening of gene knockouts. We anticipate that this technological advance will make targeted gene modification a routine practice in rice.

2. Materials

2.1. TALEN assembly

2.1.1. Reagents

1. Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene).
2. T4 DNA ligase, restriction endonucleases (NEB) or (Thermo).
3. Plasmid-Safe™ ATP-Dependent DNase (Epicentre).
4. Plasmid Miniprep kit and DNA gel Extraction kit (Axygen).

5. Chemically competent cells of *Escherichia coli* DH5α (TransGen).
6. Taq DNA polymerase (TransGen).
7. Gateway® LR Clonase® Enzyme mix (Life Tech).

2.1.2. Equipment and Consumables

1. Polymerase chain reaction (PCR) thermocycler (Biorad).
2. Environmentally controlled incubators (Eppendorf).
3. Heating water bath (Changfeng).
4. Nanodrop spectrophotometer (Thermo).
5. Standard equipment and regents for agarose gel electrophoresis (Biorad).

2.2. Protoplast isolation and transformation

2.2.1. Reagents

1. Enzyme solution: 1.5% (W/V) Cellulase RS (Yakult), 0.75% (W/V) Macerozyme R-10 (Yakult), 0.8 M mannitol (Ameresco), 10 mM MES (Ameresco) at pH 5.7, 10 mM $CaCl_2$ (Ameresco) and 0.1% BSA (Ameresco).
2. W5 solution: 154 mM NaCl (Ameresco), 125 mM $CaCl_2$ (Ameresco), 5 mM KCl (Ameresco), 2 mM MES (Ameresco) at pH 5.7.
3. WI solution: 0.5 M mannitol (Ameresco), 20 mM KCl (Ameresco), 4 mM MES (Ameresco) at pH 5.7.
4. MMG solution: 0.4 M mannitol (Ameresco), 15 mM $MgCl_2$ (Ameresco), 4 mM MES (Ameresco) at pH 5.7.
5. PEG solution: 40% (W/V) PEG 4000 (Sigma), 200 mM mannitol (Ameresco), 100 mM $CaCl_2$ (Ameresco).

2.2.2. Equipment and consumables

1. 6-Well flat-bottom plates (Nunclon).
2. Single edge razor blades (Feiying).
3. 50 mL round-bottom centrifuge tubes (Haimeng).
4. Environmentally controlled incubator (24 °C) (Yiheng).
5. Microscope, Fluorescence Microscope (Olympus).
6. 40 μm nylon meshes (BD Falcon).

2.3. PCR/RE assay

1. DNA Quick Plant System (Tiangen).
2. T4 DNA ligase, restriction endonucleases (NEB or Thermo).
3. Taq DNA polymerase (TransGen).
4. 10 mM mixed deoxynucleotides (Biodee).
5. Primer pairs (10uM) (Beijing Genomics Institute).
6. pUC-T vector (CWBiotech).
7. DNA sequencing (BGI).
8. Polymerase chain reaction (PCR) thermocycler (Biorad).
9. Standard equipment and regents for agarose gel electrophoresis (Biorad).

3. Methods

3.1. Targeted mutagenesis strategy

Rice is an ideal model plant for targeted genome engineering because of its well-annotated genome and the availability of efficient transformation systems for both protoplasts and stable plants. We have developed a simple strategy for targeted mutagenesis in rice using TALENs. Fig. 2 is a schematic representation of this strategy, which includes assembly of the TALENs by the Golden Gate method, *in vivo* assay to select those that are active, transformation of the active TALENs into rice calluses, screening of mutant plants and confirmation of genotypes. A key step in obtaining the desired mutants is selecting the most efficient TALENs for genetic transformation. We advise researchers to perform TALEN activity assays in rice protoplasts before stably transforming TALENs into rice calluses in order to minimize the cost and labor spent on subsequent tissue culture and mutant screening.

3.2. TALEN assembly

The repetitive nature of the DNA binding domains (DBDs) of TALEs makes it a major challenge to construct engineered TALENs by traditional cloning techniques; however, several TALEN assembly methods have been developed [54]. One of the most widely used is based on the Golden Gate assembly method [16]. By using type IIS restriction endonucleases, modules containing the desired RVDs can be released and assembled to create full length constructs. Considering cost and practicality in relation to the limitations of small-scale research, we recommend the reagents created by the Voytas laboratory [16]. The plasmid kit and a detailed protocol for assembly can be obtained from Addgene (http://www.addgene.org/).

Since non-matching nucleotides decrease TALE recognition and binding activity, an optional step confirming that the target sequences exist in the targeted rice variety is strongly advised. This step can help to maintain efficient TALEN activity and avoid the effects of single nucleotide polymorphisms (SNPs) in rice varieties. The complete genomic sequence or a fragment of 300–500 bp encompassing the target sequence of the relevant gene should be confirmed by PCR and DNA sequencing. The TALEN sequences can then be designed.

TAL Effector–Nucleotide Targeter (TALE-NT) 2.0 (https://tale-nt.cac.cornell.edu/), supplied by the Bogdanove laboratory, can be used to select TALEN target sites and design arrays of TALEs [55]. We routinely select TALEN targeting sequences using the following rules: (1) select RVDs according to the one-to-one correspondences: NI corresponds to A, HD to C, NG to T, and NN to G; (2) always start with a T at position 0, but do not restrict the last nucleotide to a T; (3) design three or four TALEN sites per gene; this is generally sufficient to obtain the desired mutation; (4) select TALEN sites located in the exons in the first 1/3–1/2 of the coding sequence following ATG; this is helpful in obtaining null mutations; (5) the binding sequences should consist of 15–18 bp and the spacer should be of similar length, as this facilitates FokI dimerization. Hence the complete target site can be designated as 5′-$TN^{15-18}N^{15-18}N^{15-18}$A-3′, where the left TALEN targets the 5′-TN^{15-18}-3′ on the plus strand and the right TALEN targets the 5′-N^{15-18}A-3′ on the minus strand (N = A, G, T or C). The uniqueness of the selected target sites in the rice genome should be confirmed by the same software, or by BLAST DNA sequences of TALEN binding in a related rice genome database. (6) Importantly, we strongly recommended that there should be a specific restriction enzyme site in the middle of the spacer sequence to simplify the TALEN activity assay.

The corresponding TAL effector arrays are constructed following the protocol (http://www.addgene.org/static/cms/files/Golden_Gate_TALEN_assembly_v6.pdf) provided by the kit releaser with a few modifications. A high quality TALEN plasmid library of repeats is the most important requirement, particularly for the first cycle of ligation. We suggest that the concentrations of all the plasmids be adjusted to 100 ng/μL before assembly. This method includes two ligation steps: first we ligate arrays of the first 10 repeats and arrays of the rest of the repeats separately in appropriate array vectors, but we omit sequencing of the assembled arrays because of time and cost considerations; second, the two component repeat arrays and the last half repeat are ligated correctly into the backbone vector with the truncated N△152/C63 backbone architecture (pZHY500 for the left TALE and pZHY501 for the right). After successful assembly, we sequence the complete repeats of

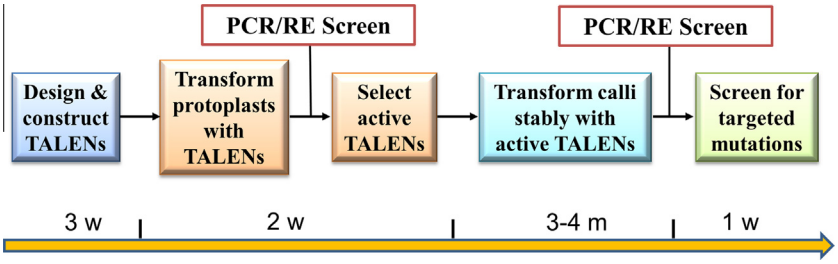

Fig. 2. Procedure for recovery of mutants generated by TALENs in rice.

the pZHY500-TALE and the pZHY501-TALE from both forward and reverse directions. Usually, sequences of no more than 18 repeats are easy to read through by overlapping the results for the two sequences.

To simplify the transfer of TALEN pairs into expression vectors with different uses, e.g. for transient or for stable genetic transformation, an appropriate Gateway entry vector plasmid is necessary. pZHY013, which contains two obligate FokI heterodimer domains separated by a T2A translational skipping sequence, is used to construct left and right TALE arrays on the same backbone [33]. In this procedure, fully assembled TALE arrays are released by XbaI/BamHI from pZHY500 or pZHY501, and subcloned into pZHY013 at the XbaI/BamHI site (for the left TALE) and at the compatible NheI/BglII site (for the right TALE). A Gateway LR reaction is performed to move the TALEN pair into the destination vector pZHY051 to construct the TALEN protoplast expression vector [33]. To construct the TALEN plant expression vector, we clone the TALEN pair into the plant binary vector pGW3, which uses the maize ubiquitin promoter to drive the overexpression of TALEN pairs in rice plants [34]. The pGW3-TALEN is subsequently transformed into *Agrobacterium* strain AGL1 using the liquid nitrogen freeze/thaw method, and positive colonies are selected on plate.

3.3. TALEN activity assays in rice protoplasts

Although surrogate assay systems, such as yeast or protoplast-based single-strand annealing (SSA) report systems [33,56], work well for assessing TALEN activities in many organisms, we believe that the transient protoplast *in vivo* assay system evaluates TALEN activity more accurately and correctly. TALEN-induced mutagenesis in rice usually involves the introduction of a heterogeneous mix of small insertions or deletions (indels) into the target sequences. Rapid and efficient monitoring of the generated mutations is a challenge. High throughput DNA sequencing of PCR products of the targeted sequence by 454 sequencing is the simplest and most efficient strategy for evaluating TALENs, but the high cost renders this method impractical for ordinary laboratories. A popular PCR/RE assay, which detects the disruption of a preselected restriction enzyme site in the spacer region, has been described for TALEN validation in *Arabidopsis* [56] and tobacco [33]. We adopted the same PCR/RE strategy [56] to analyze TALEN activity by transiently expressing TALENs in rice protoplasts. If TALENs are active at the target site, the loss of the restriction site can be measured by digesting the PCR amplicons of 300–500 bp fragments encompassing the cleavage site and the TALEN binding sites using flanking primers. The intact PCR products are subsequently sub-cloned into the sequencing vector and sequenced, and the mutation information can be used to evaluate TALEN function.

The quality of the TALEN expression plasmid DNA and rice protoplasts is very important for efficient protoplast transformation. Two aspects of the protocol should be emphasized: one is the need to use the high quality plasmids encoding TALENs at concentrations of at least 1 μg/μL; the other is to perform the PCR/RE screen only when the protoplast transformation efficiency is more than 50%.

3.3.1. Plant preparation

1. Rice cultivar: Nipponbare, Zhonghua11 or other varieties.
2. Seed sterilization: 75% ethanol prewash for 1 min, sterilization in 2.5% sodium hypochlorite for 20 min, sterile water wash 5–7 times.
3. Plant growth conditions: plate 15–20 seeds in one 1/2 MS solid medium container (diameter, 66 mm); plants will grow at 28 °C with a photoperiod of 16 h light (~150 μmol m^{-2} s^{-1}) and 8 h dark for 10–14 days.

3.3.2. Protoplast isolation

Usually protoplasts isolated from 100–120 seedlings can generate 1x10^7 cells, which can be used to transform ~20 plasmids (5×10^5 cells per transformation).

1. Bundle together the stems and sheaths of 30–40 seedlings. Use sharp razors to cut them into approximately 0.5 mm strips.
2. Immediately transfer the strips into a petri dish containing 0.6 M mannitol and incubate for 10 min in the dark at room temperature for rapid plasmolysis.
3. Remove the mannitol solution, add 50 ml of filter-sterilized enzyme solution, then transfer the strips into a 100 ml conical flask, followed by vacuum-infiltration for 30 min in the dark using a vacuum pump at −15 to −20 (in Hg). Then incubate in the dark for 5–6 h at room temperature with gentle shaking (60–80 rpm).
4. Add an equal volume of W5 solution to the conical flask, and shake by hand for 10 s to mix.
5. Collect the protoplasts into 3–4 new 50 ml round-bottom centrifuge tubes by filtering the mixture through 40 μm nylon meshes, followed by washing the strips 3–5 times with W5 solution.
6. Centrifuge at 250 g for 3 min in a swinging bucket, and remove the supernatants. Resuspend the pellets in 10 ml W5 solution per tube and collect the protoplasts into one 50 ml round-bottom tube.
7. Centrifuge at 250*g* for 3 min, remove the supernatants, and resuspend the pellets in about 4 ml MMG solution at a final concentration of 2.5×10^6 cells mL^{-1}.

3.3.3. Protoplast transformation

A polyethylene glycol (PEG)-mediated transformation method is described in this section. All the procedures can be performed in a sterile hood or in the open.

1. Transfer 200 μL protoplasts (5×10^5 cells) into a sterilized 2 mL round-bottom centrifuge tube and mix gently with 20 μg plasmid DNA (20 μL), including pZHY051-TALENs or pJIT163-GFP as positive controls, by slowly pipetting up and down.

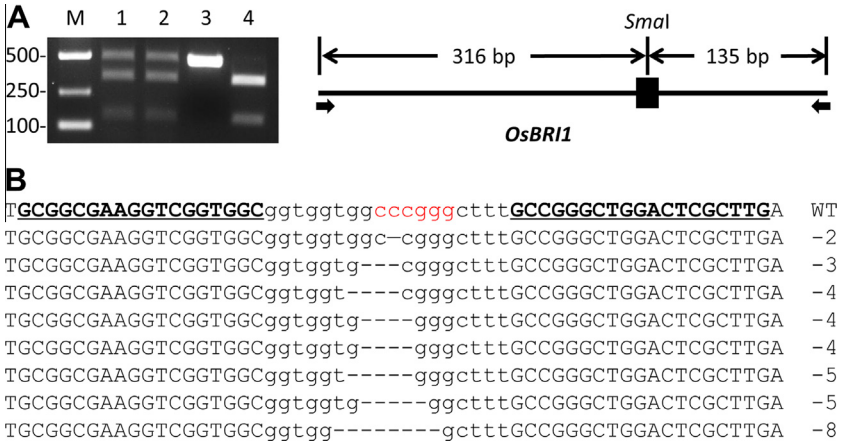

Fig. 3. PCR/RE assay for selecting active TALENs targeting the *OsBRI1* gene in rice protoplasts. (A) A representative gel for analyzing the PCR products generated from protoplast samples treated with the appropriate TALENs (lanes 1 and 2). Lanes 3 and 4 are undigested and digested wild-type controls, respectively. (B) DNA sequencing of the uncleaved bands in lanes 1 and 2. The TALEN target sites are shown in bold letters and underlined; deletions are indicated by dashes. Letters in red are SmaI sites used for the restriction digestion assay. The numbers on the side indicate the type of mutation and how many nucleotides are involved.

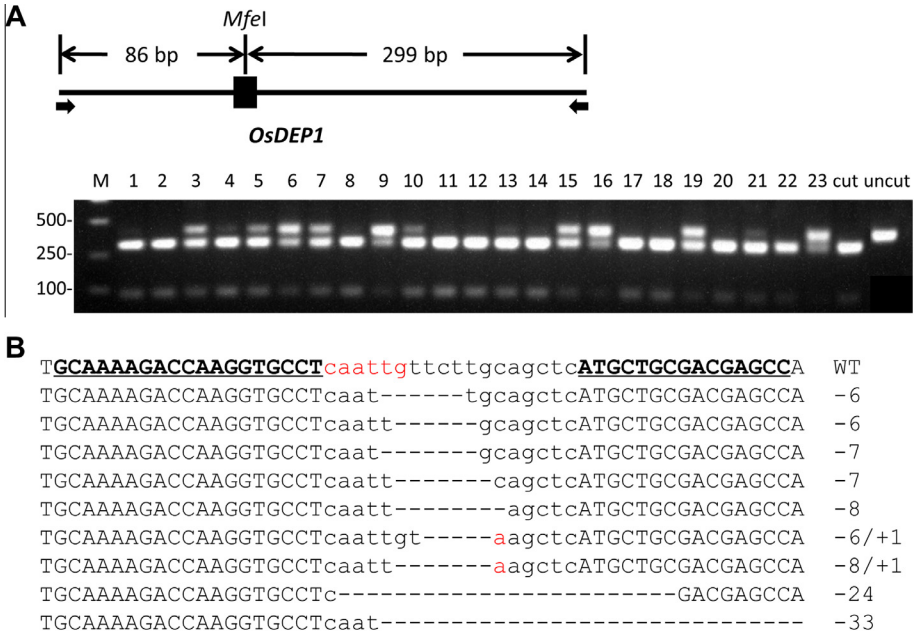

Fig. 4. PCR/RE assay for screening TALEN-induced rice mutants (targeting the *OsDEP1* gene) in transgenic plants. (A) DNA samples were extracted from leaves of each plant and PCR-amplified across the TALEN recognition sites. Each PCR amplicon was analyzed for mutations using the restriction enzyme digestion method described above for the protoplast assay. Lanes 3, 5, 6, 7, 9, 10, 15, 16, 19 and 23 are monoallelic mutants. (B) DNA sequencing of the uncleaved bands in lanes 1 and 2. The TALEN target sites are in bold letters and underlined. Deletions and insertions are indicated by dashes and red letters, respectively. Red letters are MfeI sites used for the restriction digestion assay. The numbers on the side indicate the type of mutation and how many nucleotides are involved.

2. Add 220 μL freshly prepared PEG solution and mix gently by inverting the tube several times; then incubate at room temperature for 20 min in the dark.
3. Add 880 μL W5 solution slowly to the tube; mix by inverting the tube gently by hand.
4. Centrifuge at 250*g* for 3 min, remove supernatants, then resuspended the protoplasts gently in 2 mL WI solution.
5. Transfer the protoplasts into 6-well plates, wrap the plates in aluminized foil and incubate in the dark at room temperature for 48 h.
6. Check the health of the cells by monitoring them under a microscope; the cells should appear full and round. Check the transformation efficiency by counting the number of GFP-fluorescing cells in the pJIT163-GFP positive control using a fluorescence microscope.
7. Transfer the protoplasts into a 2 mL round-bottom centrifuge tube, collect the samples by centrifuging at 12,000 rpm for 3 min; then remove the supernatants.
8. Genomic DNA extraction is carried out with a DNA Quick Plant System (Tiangen) according to the manufacturer's instructions. DNA concentrations are usually in the range of 20–40 ng/μL (30 μL elution volume).

3.3.4. PCR/RE assay

1. Digest 10 μL of the genomic DNA with the preselected restriction enzyme in a 20 μL reaction volume. This step is optional for TALEN pairs with high cleavage efficiencies.
2. Using 8 μL of the restriction digest (or 5 μL of genomic DNA) as template (in 25 μL reaction volumes), perform PCR to amplify

the chromosomal fragment encompassing the target site. We recommend primers that can amplify a 300–500 bp fragment encompassing the target site, which can be easily identified on a 1.5% EB gel after digestion.
3. Digest 10 μL of the unpurified PCR mixture with the specific restriction enzyme in a 50 μL reaction volume for 1–2 h.
4. Resolve the digested products by electrophoresis on a 1.5% agarose gel, loading the intact PCR products and completely digested products of the wild type as controls. A characteristic pattern should be observed (Fig. 3), and the mutations created by TALENs appear as undigested PCR products (Fig. 3).The frequency of TALEN-mediated mutagenesis is measured by quantifying the percentage of undigested PCR products.
5. Excise the restriction enzyme-insensitive fragments of the same size as the undigested DNA fragment, and purify them with a DNA gel Extraction kit as described by AXYGEN.

3.3.5. Confirming mutagenesis

1. Clone the mutant fragments into sequencing vector pUC-T according to the manufacturer's protocol.
2. Transform 50 μL of DH5α competent cells with 5 μL ligation mixture per reaction following the manufactures' instruction. Spread the transformed *E. coli* on LB plates with Amp (100 μg/L), IPTG(20 μg/L) and X-gal (40 μg/L) and incubate in a 37 °C incubator overnight.
3. Pick 30–50 white colonies from each plate and perform colony PCR, then select correct clones for the overnight culture.
4. Miniprep the plasmids and confirm the correct colonies by repeating the restriction enzyme digestion, but the digestion time can be shortened to 5–10 min.
5. Send the clones containing restriction enzyme-insensitive inserts for confirmation by DNA sequencing.

3.4. Generation gene knockout mutants and screening

To generate knockout mutants, *Agrobacterium*-mediated transformation of the rice cultivar Nipponbare is performed according to Hiei et al. [57]. Usually, 40–50 transgenic lines per TALEN pair are regenerated in order to obtain targeted mutants. Genomic DNA from individual hygromycin-resistant plants is extracted using a DNA Quick Plant System (Tiangen). The same PCR/RE digestion assay used in the protoplasts is applied to screen for transgenic lines (Fig. 4A). Candidate mutants are further confirmed by DNA sequencing (Fig. 4B). If the TALEN activity is efficient enough, mono allelic mutations are observed in most cases, but in some cases bi-allelic mutations can also be obtained in T_0 plants, particularly when the TALEN activities are high.

4. Conclusion

Although TALEN technology benefited from the discovery of the DNA recognition code of TALEs, which originated in a plant pathogen, the application of TALENs to plants is far behind that to other organisms. Here we present a simple and efficient protocol for performing TALEN-induced mutagenesis in rice. Using this protocol, our group has knocked out 52 rice genes and established a platform for large-scale TALEN mutagenesis. Instead of a laborious plant transformation procedure, we use a rapid, accurate and reliable protoplast transient assay, based on PCR/RE digestion, to quickly screen nuclease activity independently at each endogenous target site [34]. The active TALENs are subsequently transformed into embryonic cells of rice using the *Agrobacterium* transformation method. Mutation rates reach > 30% as measured by PCR/RE assays and by sequencing. The mutant sequences we have identified contain small deletions and insertions. Most mutations are small deletions ranging from 1 to 20 bp, and they often occur in the spacer region between the TALEN binding sites [34].

With this protocol, the PCR/RE assay cannot be applied to a target locus if no restriction site is found in the spacer region. As an alternative, a mismatch digestion enzyme such as T7 endonuclease or CEL1 might be used. In addition, large deletions can be obtained by co-transforming two pairs of TALENs targeting the same chromosome by the particle bombardment method. Although off-target effects of TALENs have not so far been reported in rice, we nevertheless suggest testing carefully for off-target mutagenesis at potential homologous sequences, if possible. Also whole genome sequencing may be necessary to confirm the linkage between artificial genotyping and TALEN mutants in some important cases.

In summary, TALEN technology is a powerful and adaptable approach to genome editing in plants. TALEN mutagenesis in rice provides an efficient and robust way to create mutants for reverse genetics research.

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