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ORIGINAL RESEARCH

TALEN-Mediated Homologous Recombination Produces Site-Directed DNA Base Change and Herbicide-Resistant Rice

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ABSTRACT

Over the last decades, much endeavor has been made to advance genome editing technology due to its promising role in both basic and synthetic biology. The breakthrough has been made in recent years with the advent of sequence-specific endonucleases, especially zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) guided nucleases (e.g., Cas9). In higher eukaryotic organisms, site-directed mutagenesis usually can be achieved through non-homologous end-joining (NHEJ) repair to the DNA double-strand breaks (DSBs) caused by the exogenously applied nucleases. However, site-specific gene replacement or genuine genome editing through homologous recombination (HR) repair to DSBs remains a challenge. As a proof of concept gene replacement through TALEN-based HR in rice (*Oryza sativa*), we successfully produced double point mutations in rice acetolactate synthase gene (*OsALS*) and generated herbicide resistant rice lines by using TALENs and donor DNA carrying the desired mutations. After ballistic delivery into rice calli of TALEN construct and donor DNA, nine HR events with different genotypes of *OsALS* were obtained in T_0 generation at the efficiency of 1.4%−6.3% from three experiments. The HR-mediated gene edits were heritable to the progeny of T_1 generation. The edited T_1 plants were as morphologically normal as the control plants while displayed strong herbicide resistance. The results demonstrate the feasibility of TALEN-mediated genome editing in rice and provide useful information for further genome editing by other nuclease-based genome editing platforms.

KEYWORDS: TALEN; Genome editing; Homologous recombination; Gene replacement; Site-directed mutagenesis; Acetolactate synthase; Herbicide resistance; Rice

INTRODUCTION

Genome editing, a process of precisely targeting and modifying predetermined genomic DNA sequences, is always challenging in the last decades though much effort has been made to artificially engineer sequence-specific endonucleases. Artificial endonucleases manipulate the cellular repair mechanisms through controlling the location and quantity of DNA double-strand breaks (DSBs). Not until the advent of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs)-guided nucleases (e.g., Cas9 and Cpf1) did site-directed mutagenesis or gene replacement become much easier (Smith et al., 2000; Christian et al., 2010; Li et al., 2011; Jinek et al., 2012). The rapid development of those powerful tools has been providing promise to rapid advance of basic and applied scientific research. However, genomic editing or gene replacement still remains a challenge in most of eukaryotic organisms.

Transcription activator-like effectors (TALEs), a large group of highly conserved type III effector proteins, exist in *Xanthomonas* spp. and can be translocated into host cells by the bacterial type III secretion system (White et al., 2009). Different bacterial strains contain a varying number of TALEs, but majority of those

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effectors contain an N-terminal bacterial secretion and translocation signal, central DNA binding domain, and C-terminal eukaryotic nuclear localization signals (NLSs) and a highly conserved acidic activation domain (AD) (Boch and Bonas, 2010). The DNA binding domain composes of an array of tandem repeats of 34 amino acids; and the repeats consist of nearly identical amino acids except those at the position 12th and 13th, which are termed Repeat Variable Di-residues (RVDs). Collectively, the number of TALE repeats (ranging from 13 to 33) and composition of RVDs determine the specificity for DNA binding (Boch et al., 2009; Moscou and Bogdanove, 2009). The simple TALE/DNA recognition code is best described as one repeat corresponding to one nucleotide and one type of RVD preferentially recognizing one type of nucleotide of target DNA. In a practical means, four types of repeats that predominantly occur in nature are the repeats with RVDs HD, NI, NG and NN that recognize nucleotides cytosine (C), adenine (A), thiamine (T), and guanine (G) or A, respectively.

With high specificity of DNA recognition and relatively low dependence on genomic context of target DNA, TALE repeats, especially the four predominant ones, have been adopted to engineer DNA binding domains and assemble into sequence-specific endonucleases by fusing to the FokI nuclease domain (Christian et al., 2010; Li et al., 2011). Accordingly, various modular assembly methods have been developed to engineer TALENs, including the most widely used golden-gate cloning method, automated solid-phase high-throughput method, PCR dependent or ligation independent subcloning techniques. TALEN genes can either be made in laboratories, or be available commercially (Cermak et al., 2011; Reyon et al., 2012; Schmid-Burgk et al., 2013). TALENs have been successfully used mostly for targeted mutagenesis in more than 25 organisms including yeast, animal, plant and human cells (Weeks, et al., 2016).

There are two DSB repair pathways in eukaryotic organisms, namely non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is a process that directly joins the broken ends without homologous sequences or utilizes exposed micro-homologous ends to guide joining. The breakage can be precisely resealed or imprecisely repaired leading to small insertions or deletions (Mladenov and Iliakis, 2011). HR uses homologous region from donor DNA as a template to repair the breakage (Fig. 1) (San Filippo et al., 2008). In this case, the repair process is exploitable to generate site- or sequence-specific DNA mutation through sequence replacement, or to precisely integrate genes of interest such as florescent markers into target sites for various research and application purposes. However, relatively few studies have been reported for HR-mediated genome modifications largely due to the low intrinsic recombination efficiency in eukaryotes. For example, only a few cases have been reported in plants for ZFN or CRISPR (Wright et al., 2005; de Pater et al., 2009, 2013; Townsend et al., 2009; Li et al., 2013). In those studies, DNA was delivered through *Agrobacterium*-mediated floral dip in *Arabidopsis* or transfected into tobacco protoplasts.

Acetolactate synthase gene (*ALS*) encodes a protein that as an enzyme is involved in the biosynthesis of branched-chain amino acids valine, leucine and isoleucine (Chipman, et al., 1998). The gene product, acetolactate synthase (also known as acetohydroxy acid synthase or AHAS) represents a major target for development of distinct classes of herbicides that inhibit its enzymatic activity and hence block the synthesis of these amino acids. Important herbicides such as chlorsulfuron and bispyribac-sodium (BS) have been developed to control a wide spectrum of grass and dicot weeds. To generate rice plant tolerant to the pyrimidinyl carboxy herbicide bispyribac-sodium, prior studies had identified an *OsALS* mutant gene ($OsALS^R$) that could tolerate BS through rice tissue culture and regeneration (Osakabe, et al., 2005; Kawai, et al., 2007). Two point mutations in $OsALS^R$ are crucial for BS resistance: a G to T missense change leading to the amino acid tryptophan (TGG) to leucine (TTG) at the position 548 (W548L) and a second G to T missense change resulting in the substitution of serine (AGT) with isoleucine (ATT) at the position 627 (S627I) (Shimizu et al., 2005). The double point mutations increase BS tolerance compared to single mutation. BS tolerant rice plants were reproduced by introducing the double point mutations to *OsALS* endogenous loci at a very low rate through conventional gene targeting without the aid of DSB-causing endonucleases (Endo et al., 2007). Additionally, this herbicide tolerant *ALS* has been successfully used as a selection marker for plant transformation (Endo et al., 2012).

In the present study, as a proof of concept to demonstrate the feasibility of using TALENs for gene replacement in plant, we constructed a single construct that contained both the *OsALS*-targeting TALEN expression cassettes and the donor DNA fragment for the double point mutations. Through ballistic delivery, this construct yielded high efficiency of rice transformation and up to 6.3% of gene edits in three independent experiments. The HR-mediated mutations were able to pass on to the T_1 generation progeny as tested. Furthermore, the T_1 plants showed strong BS resistance and were morphologically normal compared to the parent line. No HR event was detected in two sets of negative control experiments.

RESULTS

Rice ALS variant with two point mutations confers herbicide resistance in transgenic rice

The rice acetolactate synthase gene (*OsALS*) is transcribed from a single exon in chromosome 2 (*Os02g30630*) and its cDNA clone is 2279 bp (NCBI accession No., NM_001053466). To confirm the prior report that showed double point mutations (W548L and S627I) in *OsALS*, namely W548L/S627I or referred to here as $OsALS^R$ allele, conferred strong resistance to bispyribac-sodium (BS), a pyrimidinyl carboxylate type herbicide (Kawai, et al., 2007), we reconstructed the mutations based on the *ALS* gene amplified from the BS sensitive rice cultivar Kitaake and introduced the mutated gene ($OsALS^R$) into Kitaake through *Agrobacterium*-mediated gene transfer (Fig. 2A). Three $35S{:}OsALS^R$ transgenic rice lines were chosen for detailed characterization of $OsALS^R$. When grown in rice solution supplemented with