Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes

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ABSTRACT

Recent studies indicate that the DNA recognition domain of transcription activator-like (TAL) effectors can be combined with the nuclease domain of FokI restriction enzyme to produce TAL effector nucleases (TALENs) that, in pairs, bind adjacent DNA target sites and produce double-strand breaks between the target sequences, stimulating non-homologous end-joining and homologous recombination. Here, we exploit the four prevalent TAL repeats and their DNA recognition cipher to develop a ‘modular assembly’ method for rapid production of designer TALENs (dTALENs) that recognize unique DNA sequence up to 23 bases in any gene. We have used this approach to engineer 10 dTALENs to target specific loci in native yeast chromosomal genes. All dTALENs produced high rates of site-specific gene disruptions and created strains with expected mutant phenotypes. Moreover, dTALENs stimulated high rates (up to 34%) of gene replacement by homologous recombination. Finally, dTALENs caused no detectable cytotoxicity and minimal levels of undesired genetic mutations in the treated yeast strains. These studies expand the realm of verified TALEN activity from cultured human cells to an intact eukaryotic organism and suggest that low-cost, highly dependable dTALENs can assume a significant role for gene modifications of value in human and animal health, agriculture and industry.

INTRODUCTION

Technologies for precise and efficient gene editing in living cells hold great promise in both basic and applied research, including therapeutic interventions for genetic diseases. These technologies exploit the ability of endonucleases to cause chromosomal double-stranded DNA breaks (DSBs) and stimulate the subsequent breakage repair mechanisms in living cells (1–3). The two widely conserved, major repair pathways in eukaryotes are non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ repair often results in mutagenic deletions/insertions and substitutions in the targeted gene. DSBs also stimulate HR between the endogenous target gene locus and an exogenously introduced homologous donor DNA carrying desired genetic alterations (4–6). At the forefront of these methods are custom-designed zinc-finger nucleases (ZFNs), which are hybrid proteins derived from the DNA binding domains of zinc-finger (ZF) proteins and the non-specific cleavage domain of the endonuclease FokI. However, despite their promise, widespread adoption of ZFNs is hampered by a bottleneck in custom-engineering ZFs with high specificity and affinity for the DNA target sites (7,8). Further, ZFN utility is somewhat limited by the number and location of potential target sites within a genome. Alternative strategies that overcome limitations in current technologies for targeted genome editing could greatly accelerate adoption of artificial nucleases for efficient gene disruption and gene replacement in a variety of heretofore recalcitrant eukaryotic organisms.

ZFN efficacy depends almost solely on the DNA binding specificity of their ZF domains (9), which theoretically can be supplanted by any high-fidelity DNA
binding domain (10–12). Transcription activator-like effector (TALE) proteins, a large group of bacterial plant pathogen proteins, have emerged as alternatives to ZF proteins. TALE proteins contain a varying number of centrally located tandem 34-amino-acid repeats that mediate binding to a specific DNA target sequence, referred to as the effector binding elements (EBE). Each repeat is nearly identical except for two variable amino acids at positions 12 and 13, known as repeat variable di-residues (RVD). Polymorphism in the number of repeats (a range of 13–33) and in the RVD composition collectively determines the DNA binding specificity of individual TALE proteins. Remarkably, recognition of a specific DNA sequence is based on a fairly simple code wherein one base of the DNA target site is recognized by the RVD of one repeat (i.e. one repeat/one nucleotide). The sequential repeat arrangement in a single TALE protein thus specifies the contiguous DNA sequence that will be bound by that TALE protein (13,14). TALE recognition studies also reveal a preference for certain RVDs over others in recognizing a particular nucleotide. In most cases, the RVDs asparagine and isoleucine (NI), histidine and aspartic acid (HD), asparagine and glycine (NG) and two asparagines (NN) combine to recognize the nucleotides ‘A’, ‘C’, ‘T’ and ‘G’, respectively (13,14). The prevalence of these four RVDs in the native TALEs made it possible to use them exclusively to de novo synthesize or assemble TALE repeat arrays of up to 13 repeat units to target DNA sequences in plant and human cells (15–17). The addition of FokI nuclease domains to the C-termini of two paired synthetic TAL effectors (as in ZFNs) should allow for highly gene-specific gene targeting. Because of the modular nature of TALEs and the potential to use long DNA recognition sites, custom-made designer TALE nucleases (dTALENs) may overcome limitations of current ZFN technologies and may significantly advance the use of targeted genome editing for challenging long-term opportunities such as therapeutic repair of genetic diseases. Toward this end, we and others have recently shown that TALEs can be linked with FokI nuclease domain to direct targeted cleavage of DNA containing a specific EBE (17–19). More recently, Miller et al. (20) demonstrated TALEN-mediated editing of endogenous genes in cultured human cells.

Here, we report development of a modular assembly technology for custom-engineering dTALENs and characterization of 10 such dTALENs for targeted gene modification. The method uses four basic repeats with RVDs of NI, NG, NN and HD to generate 48 ready-to-use modules of single-TAL repeat units that can be used to assemble up to 23 repeat units in any predetermined order. The modular assembly method is simple, fast and inexpensive and can be performed in most academic or industrial molecular biology laboratories. Remarkably, all 10 dTALENs demonstrated efficient gene knockout and/or gene replacement in tests with three different chromosomal genes in yeast (Saccharomyces cerevisiae). The high success rate and facile synthesis of potent dTALENs against a variety of chromosomal targets further establishes dTALENs as an emerging and viable technology for precise gene modification in living cells. As previously practiced, all materials described in the present study will be provided to other laboratories upon request.

MATERIALS AND METHODS

Yeast strains and growth conditions

Yeast strains YPH499, YPH500 and RFY231 as previously described (18,21) were grown in nutrient medium YPD or synthetic complete medium (SC) lacking the appropriate nutrients. 5-fluoroorotic acid (5-FOA) was used at 0.1% in SC medium as described (22). α-aminoadipate (α-AA) was used at 0.2% in SC medium lacking the normal nitrogen source, but containing a small amount of lysine (30 mg/l) (23). The adenine limited medium is SC medium containing a limited amount of adenine (10 mg/l) and lacking leucine and histidine (24).

dTALEN constructs

Four repeats, each encoding the RVD of NI, NG, HD or NN from AvrXa7, were used as the ‘core’ repeats. [More recently, NK has been substituted for NN in the recognition of G nucleotides based on the observations of Miller et al. (20).] Using a combination of 12 forward and 11 reverse primers (Supplementary Table S1), 12 repeat sets were constructed from the 4 ‘core’ repeats. For construction of the first 8–mer repeat array, combinations of the PCR primers were: TAL-Sph-F and TALccct-R, TALccct-F and TALccct-R, TALctct-F and TALctct-R, TALctct-F and TALctct-R, TALctct-F and TALctct-R, TALctct-F and TALctct-R, TALctct-F and TALctct-R, TALctct-F and TALctct-R, TALctct-F and TAL/Pst-R for repeat Set 1–8, respectively. For ligation of the second and the third 8–mer repeat arrays, two additional repeat sets each corresponding to Set 1 were constructed by using primers TAL/Pst-F&TALctct-R and TAL/Bsr-R&TALctct-R, respectively; another two repeat sets corresponding to Set 8 were also constructed by using primers TALctct-R and TAL/Bsr-R, and Tailcact-F&TAL-Sph-R, respectively. In total 12 repeat sets were generated and then individually digested with BsmBI. Based on the base entity and order of the preselected target DNA sequence (e.g. any sequence combination of 22 bp long), one repeat from each of eight repeat sets was sequentially selected for one ligation reaction to construct the 8–mer repeat array. Each set of ligated DNA was directly cloned into pGEM-T for sequencing. Once confirmed, the first 8–mer array was digested with SphI and PstI, the second 8–mers with PstI and BsrGI, and the third array with BsrGI and Sall. The three purified DNA fragments were ligated into pSK/AvrXa7 (18) that was digested with SphI and Sall, resulting in pSK/dTALE plasmids. The repeat regions of individual dTALEs were used to replace the TALE repeat domain of AvrXa7 in pCP3M-AvrXa7-FN or pCP4M-AvrXa7-FN (18), resulting in chimeric genes encoding the fusion of the full-length dTALE and the C-terminal FokI homodimeric cleavage domain. The expression level of these nuclease genes should be moderate due to the low copy number (about one copy per cell) of the centromeric plasmids pCP3M and pCP4M and the strong promoter from the translation elongation factor 1α gene (25).
Yeast SSA assay for dTALEN activity

The individual target sites were constructed into pCP5 (18) in a tail-to-tail (3' to 3') orientation with AvrXa7 EBE. The oligonucleotide sequences for all TALEN EBEs were provided in Supplementary Table S2. AvrXa7 EBE (×7-EBE-F&R) was cloned into the PstI and SpeI sites of pCP5. The EcoRI site immediately upstream of SpeI site enabled all other TALEN EBEs to be cloned individually between EcoRI and SpeI sites, resulting in the reporter plasmids for individual TALENs in combination with AvrXa7-FN. The assay for each individual TALEN paired with AvrXa7-FN was performed in a manner similar to that described (18). The assays were performed in triplicate.

Targeted gene disruption of URA3, LYS2 and ADE2 in yeast

The URA3 gene (ura3–52) that was insertionally inactivated by the transposon, Ty1, in YPH500 was restored to a functional URA3, resulting in strain YPH500a. Similarly, the target sequences for ZFNs (Zif268 and BCR-ABL) and for TALENs of AvrXa7 and PthXo1 were individually integrated into the URA3 gene immediately downstream of the start codon and used to restore the ura3-52 mutant, resulting in strain YPH500b and YPH500c, respectively. YPH500b was transformed with plasmids pCP3M/Zif268-FN and pCP4M/BCR-ABL-FN. DNA fragment for the ZF protein Zif268 was PCR amplified from pMal-Zif268 (kindly provided by David J. Segal) using primers Zif268-F and Zif268-R and cloned in frame with FokI cleavage domain in pCP3M. Construct pCP4M/BCR-ABL-FN was described previously (18). YPH500c was transformed with pCP3M/AvrXa7-FN and pCP4M/PthXo1-FN, two plasmids previously described (18). YPH500a was transformed with plasmids expressing the paired dTALENs U3a-L and U3a-R, U3b-L and U3b-R, or with the plasmids lacking a nuclease gene as control. The transformed cells were grown in SC media lacking histidine and leucine for 5 days, then plated on SC medium supplemented with either 5-FOA (for pΔura3) or YPD medium supplemented with 200 mg/l of G418 (for pΔura3:Kan), the duplicated cells were in parallel plated on SC medium or YPD medium to determine plating efficiency. See Supplementary Data for additional details.

Cell growth assay

YPH500 cells were transformed with individual plasmids as indicated in the text. Three single colonies for each plasmid were picked and grown to a concentration of OD600 = 1.0. The cells were serially diluted and applied to appropriate solid medium for growth, which was observed daily for 5 days.

Solexa sequencing and data analysis

The genomic DNA from each of five yeast strains derived from YPH500, which is congenic with S288C (26), was extracted as described by Philippsen et al. (27). The DNA processing and sequencing were performed according to manufacturer’s instruction for the Illumina/Solexa Genome Analyzer II at the Iowa State University DNA facility. The Illumina short reads for each strain were aligned to the yeast reference genome S288C to assemble each genome using the BWA software (28). The consensus sequences and polymorphisms among the five sequenced strains and S288C were delineated using SAMtools (29).

RESULTS

Tractability of yeast for testing gene modification by TALENs

We chose yeast, a classic eukaryotic model for studies of HR (30), as a platform to develop and test TALEN-based technologies for targeted gene modification based on the recent breakthroughs in the area of TALE research (13,14,17,18). First, we determined if the yeast chromosome, which has not been subjected to genome modification using any artificial nucleases, is tractable for TALE nuclease-based modification. For this experiment, the EBEs for AvrXa7 and PthXo1 were precisely integrated in-frame between the first and second codons of the yeast URA3 gene on chromosome 5. In parallel, DNA target sequences for the known ZFNs Zif268 and BCR-ABL were inserted into the identical URA3 gene site for comparison of activities conferred by these two types of nucleases (Figure 1 and Supplementary Figure S2). The resulting yeast strains were prototrophic in uracil-free medium and sensitive to 5-FOA, indicating that the chimeric URA3 genes were functional and intact. Yeast strains bearing the chimeric URA3 genes were transformed with plasmids expressing the paired TALENs or ZFNs, grown for 5 days, plated on medium containing 5-FOA to select cells with an inactivated URA3 gene. Approximately 0.9% of cells (out of ~10⁶ cells) expressing paired TALENs produced 5-FOA resistant colonies while
0.3% of cells expressing paired ZFNs yielded 5-FOA resistant colonies. In contrast, no 5-FOA resistant colonies were observed among $10^6$ cells containing plasmids lacking a functional nuclease gene (a ratio of <0.0001%). Sequenced PCR products from the relevant alleles revealed all of the selected 5-FOA-resistant clones harbor mutations (insertions and/or deletions that caused frame shift in the $URA3$ genes) at the nuclease target sites (Figure 1 and Supplementary Figure S2). These results established similar gene disruptions and comparable mutation rates elicited by ZFNs and TALENs targeted against genes in a native yeast chromosomal environment.

Modular dTALEN assembly

To fully realize the potential of TALENs, they must be custom-engineered to target any chromosomal DNA sequence of interest. By exploiting the repeat homology and the unique recognition sequence of the type II restriction enzyme BsmBI within each repeat of AvrXa7 or any TALE, we developed a method to assemble repeat domains in an exact predetermined order to recognize a specific DNA sequence in any gene of choice from any organism. Briefly, four AvrXa7 ‘core’ repeats whose coding RVDs each recognize one of four nucleotides (i.e. NI, NG, NN and HD, respectively, for A, T, G and C) were used to construct independent modules (single repeats) whose 5’- and 3’-ends were designed to form a unique 4-bp overhang with single-base polymorphism after digestion with BsmBI. The BsmBI site is immediately downstream of codons 18 and 19 of each repeat and BsmBI cleaves these two triplets into a 5’-overhang of 4-bp at each end. The 18th and 19th codons are GCG CTG and can be modified into eight variant triplets GC(A, T, G or C) (T or C) TG (Figure 2A). Therefore, combinations of eight or fewer such overhangs, one at each end of a single repeat, were created without altering the encoded amino acids. This allowed the ordered ligation of eight or fewer repeat modules in any predetermined sequence for construction of sub-arrays of repeats (Figure 2B). Each sub-array was cloned into the cloning vector pGEM-T and sequenced to confirm the correct number and order of the repeats. Similarly, multiple sub-arrays (two and three in this study) were further assembled to match the order of nucleotides at the preselected genomic site. The AvrXa7-FN nuclease (18) lacking its repeat domain was used as the scaffold for the assembled repeat domains, resulting in a finished dTALENs (Figure 2C).

To test the feasibility of our approach, we selected five distinct dual target sites (two in $URA3$, two in $LYS2$ and...
one in ADE2) based on the criteria: (i) 'T' preceding each target sequence; (ii) avoiding G-rich blocks; and (iii) 17–20 bp spacer between the two inverted EBE target sites. Accordingly, 10 dTALENs were synthesized for gene targeting based on the preselected DNA coding sequences of the three yeast genes (Figure 3A). All 10 dTALENs were expressed in yeast to levels comparable to those of hybrid nucleases made from PthXo1 or AvrXa7, except U3b-L which had somewhat lower expression (Supplementary Figure S3).

To test the function of newly synthesized dTALENs and to reveal their relative DNA cleavage activity, we modified a transient and plasmid-borne single-strand annealing (SSA) assay (31,32) as a facile analytical tool. This method uses a yeast plasmid carrying a lacZ gene divided into upstream and downstream portions by insertion of two opposing EBEs, one recognized by a proven AvrXa7 TALEN (18) and the other EBE recognized by a candidate dTALEN. The separated lacZ fragments share at the 3'-end of the upstream fragment and the 5'-end of the downstream fragment a 125 bp segment of identical lacZ sequence. If a functional dTALEN is co-expressed in yeast cells with the AvrXa7 TALEN along with the target lacZ gene, it will bind to its target EBE sequence adjacent to the AvrXa7 TALEN and, thereby, create a DSB (Figure 3B). The duplicated lacZ sequences are thus available for HR and restoration of an intact lacZ gene. The amount of β-galactosidase activity produced by

Figure 2. Design and modular construction of dTALENs. (A) TALEN repeat gene sets for modular construction of multi-repeat TALEN genes. Each set contains four single-repeat genes each encoding one of the four 'core' TALEN repeat modules containing NI, NG, NN and HD RVDs with binding specificity for A, T, G and C nucleotides, respectively. The core repeats in each set (boxed) contains a 5'- and 3'-terminal unique to that particular set—as listed in the inset table. For construction of a TALEN repeat array recognizing a specific 8 nt DNA recognition sequence (e.g. AGGTACTC), an NI repeat gene is selected from Set 1, an NN repeat gene from Set 2, an NN repeat gene from Set 3, an NG repeat gene from Set 4, etc. The 5'- and 3'-terminal regions are designed such that BsmBI digestion results in generation of 5'- and 3'-base overhangs. Because of the unique complementarity of 3'-overhangs from Set 1 genes with the 5'-overhang of genes from Set 2, the complementarity of the 3'-overhangs of Set 2 with 5'-overhangs from Set 3, etc., the annealing and ligation of overhangs results in one, and only one, ordered alignment of the eight repeat genes that, when translated, will specifically recognize and bind (in the present example) the AGGTACTC DNA recognition sequence. (B) Once two (or three) such blocks of repeats are constructed, they are combined in a similar ordered fashion to create a TALE repeat region that is capable of binding a specific 16 (or 24) bp target site. (C) The assembled TALE repeat domains are cloned into the TALEN repeat-deficient pAvrXa7-FN scaffold to create a candidate dTALEN.
the transformed yeast cells thus provides a measure of the amount of DNA cleavage supported by the candidate dTALEN. We initially tested this assay by first pairing the proven AvrXa7-FN TALEN with another proven TALEN, PthXo1-FN (18). The activity of this TALEN pair provides a standard against which the activity of any candidate dTALEN can be judged (Figure 3C). The 10 newly produced dTALENs, each designed to recognize a specific 17–23 base DNA sequence in different yeast genes, were all found in the yeast SSA tests to function nearly as well or better than the standard AvrXa7/PthXo1 TALEN pair (Figure 3C).

Efficient gene modification by dTALEN-induced NHEJ and HR

As a final evaluation of the function of the five pairs of dTALENs, we tested their ability to elicit site-specific DNA alterations at the preselected target sites in the URA3, LYS2 and ADE2 genes, which all have easily scored knock-out phenotypes. Yeast cells were transformed with individual pairs of dTALEN-expressing plasmids and grown for 5 days on SC medium to allow accumulation and activity of the heteromeric dTALEN pair. Two yeast cultures were transformed separately with one or the other pair of dTALENs targeting the URA3 gene and plated for 5-FOA selection of cells lacking a functional URA3 gene. Likewise, two yeast cultures were transformed separately with one or the other pair of dTALENs targeting the LYS2 gene and plated for α-aminoadipate (α-AA) selection of cells with LYS2 gene mutations. URA3 and LYS2 mutants were obtained at a rate of $\sim 10^{-4}$ to $10^{-2}$ mutants/total cells (Figure 4A). Yeast cells transformed with the dTALENs pair targeting the ADE2 gene were plated on medium containing limiting adenine concentrations that result in the formation of pink colonies if a functional ADE2 gene is not present. Pink colonies appeared with a frequency of 0.15% (Figure 4A). In contrast, $\sim 10^6$ yeast cells carrying

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Figure 3. Yeast SSA assay of modularly assembled dTALENs targeting three endogenous yeast genes. (A) RVD sequences within repeat modules of 10 custom-synthesized dTALENs. N* represents dTALEN repeat modules with the 13th amino acid missing. (B) Schematic of the yeast SSA assay for measuring dTALEN activity based on plasmid-borne HR. Individual candidate dTALENs are assayed in combination with AvrXa7-FN for their ability to stimulate the recombination between the duplicated regions of LacZ gene (hatched boxes), leading to formation of a functional lacZ gene. (C) Activities of individual dTALENs in creating DSBs as detected in a β-galactosidase assay. Control denotes the β-galactosidase activity (<5U) of yeast cells lacking a functional TALEN gene. Error bars denote SD; n = 3.
plasmids lacking a functional dTALEN gene pair yielded no colonies resistant to 5-FOA or α-AA or with a pink color. Sequence analysis of PCR-amplified genomic DNA from the relevant target sites in several putative URA3, LYS2 and ADE2-gene knock-out mutants revealed that all alleles harboured mutations at the dTALEN target sites as expected. A high proportion of the mutated loci contained deletions in a range from 1 to 75 bp (Figure 4B and Supplementary Figure S4).

One experimentally and practically important virtue of DSBs caused by agents such as ZFNs (3) is that they increase rates of recombination between the DNA sequences within a broken gene and homologous endogenous or exogenously-supplied DNA sequences, which enables powerful gene replacement research opportunities. To determine if dTALEN-mediated DSBs stimulate HR, we targeted the URA3 gene for breakage with artificial dTALEN pairs in the presence of two different exogenously supplied DNA fragments, one containing a URA3 gene interrupted by a neomycin phosphotransferase II (NPTII) expression cassette and the other a DNA fragment with the URA3 ORF deleted. Both fragments...
contained at their 5’ ends a 0.5 kb segment homologous to the 5’ end of the URA3 gene and, at their 3’ ends, a 0.2 kb segment from the 3’ end of the URA3 gene. Yeast cells of YPH500c, a strain containing the functional URA3 with the integrated EBEs for AvrXa7 and PthXo1, were transformed with one set of three different paired TALENs (i.e. U3a-L and U3a-R, U3b-L and U3b-R, and AvrXa7-FN & PthXo1-FN) and one of the two modified URA3 gene constructs. The transformants were plated after 5 days of incubation on either selective medium containing 5-FOA (deleted URA3 construct) or medium containing the neomycin-like antibiotic, G418 (NPTII interrupted URA3). Cells transformed with TALENs and the URA3 ORF deletion construct (DURRA3) showed frequencies of 5-FOA-resistant colonies in the range of 4.5–27%. The negative control [transformed with donor DNA and plasmids lacking a nuclease gene (Empty Plasmid)] yielded 5-FOA-resistant colonies at a rate of 0.01% (Figure 4C).

The frequency of gene replacement for cells transformed with the NPTII-interrupted URA3 gene construct (NPTII) was in the range of 9–34% with the negative control (Empty Plasmid) displaying only ~0.1% gene replacement activity (Figure 4C). Overall, the enhancement of TALEN-induced gene replacement was between 100- and 2700-fold higher than the control. The scale and consistency in the stimulation of HR by dTALENs suggest they have the potential to promote HR when used in eukaryotic cells that lack other sufficiently robust mechanisms to facilitate HR.

**Genome-wide undesired mutations caused by TALENs**

Some ZFNs have been found to be associated with toxicity in living cells (33–39). Whether such effect also exists for TALENs is unknown. To test the possibility, yeast cells were transformed with plasmids encoding six pairs of TALENs (one pair of native TALE-derived nucleases and five pairs of synthetic dTALENs targeting sequences in a size range between 17 and 27 bp) and one pair of known ZFNs [Zif268 and BCR-ABL with target sequences of 9 bp (33,40)]. The transformed cells were grown in SC medium lacking leucine and histidine. During 5 days of growth, TALEN-expressing yeast cells displayed no distinct phenotype in terms of cell viability and proliferation compared to the control, as did yeast strains with ectopic expression of paired ZFNs (Figure 5). Yeast cells expressing individually each of the eight introduced nuclease genes showed similar results (Supplementary Figure S6). The results indicate the lack of 5-FOA-resistant colonies in the range of 4.5–27%.

The negative control [transformed with donor DNA and plasmids lacking a nuclease gene (Empty Plasmid)] yielded 5-FOA-resistant colonies at a rate of 0.01% (Figure 4C). Overall, the enhancement of TALEN-induced gene replacement was between 100- and 2700-fold higher than the control. The scale and consistency in the stimulation of HR by dTALENs suggests they have the potential to promote HR when used in eukaryotic cells that lack other sufficiently robust mechanisms to facilitate HR.

**Potential genome-wide undesired effects of TALENs.**

Five strains, including the parental strain YPH500 and four mutants, were chosen to investigate the occurrence of unintended mutations in addition to the site-specific mutations at the URA3 locus mediated by the respective nucleases. The four mutant strains included one that contained a deletion in the chimeric URA3 gene at the integrated EBE site targeted by the paired nucleases of natural TAL effectors AvrXa7 and PthXo1, one that contained an insertion in the chimeric URA3 gene with integrated target sequences for the ZFNs Zif268 and BCR-ABL, and the other two that each contained a deletion mutation in the wild-type URA3 genes induced by the paired dTALENs U3a-L&-R and U3b-L&-R, respectively. The five strains were sequenced using Illumina/ Solexa Genome Analyzer II, and their genomes were assembled with coverage depths [(number of reads \times average read length) / (size of genome)] in a range from 135 to 170 \times (sequences available upon request). The assembled genomic sequence of each mutant strain was first screened for possible mutations at the sites that matched or loosely matched (defined as at least two-thirds of match as the cut-off) either intended sub-dual target sequence for the respective nucleases (Supplementary Figure S5). No mutations were found at those locations other than the
site-specific mutations mediated by the respective pairs of TALENs and ZFNs in each genome (data not shown). However, alignment of the genomes of the individual nuclease treated strains with that of parental strain revealed a few mutations in each strain (in a range from 3 to 5 mutations) (Supplementary Table S4). These mutations were almost all nucleotide substitutions instead of predominant deletions/insertions at the nuclease target sites and they, therefore, were highly likely to be simultaneous mutations which might or might not be associated with the nuclease treatment.

**DISCUSSION**

We have developed a simple, cost-effective method to assemble functional TALE DNA binding domains, combine them with a nuclease module and confirm their activity against specific gene targets in vivo. Using this method, dTALENs were readily produced and rapidly validated using a facile yeast SSA assay. Importantly, these dTALENs were then demonstrated to function as pairs to mediate efficient gene disruption by NHEJ and gene replacement by HR at specific yeast chromosomal loci.

The DNA binding domain encoded by a TALE gene is modular by nature, having 13–33 RDV units that bear a simple code for target site recognition (13, 14). However, the high-repeat homology imposes technical difficulty when using PCR-based de novo gene synthesis methods (42) for construction of the lengthy repeat arrays required for high-level DNA specificity. To simplify gene synthesis and reduce dTALEN production costs, a modular assembly technique was developed. TALE repeat domains have been assembled from the single-RVD coding repeats to bind DNA targets predicted by the cipher; but they were assembled in a random way, not in a predetermined order and not based on the target DNA sequence (13). Our method involves creation of 48 ready-to-use modules and their assembly into repeat arrays in a controlled order based on any user chosen target DNA sequences (Figure 2). The ready-to-use modules can also be adapted for high-throughput dTALEN synthesis. By manipulating the combination of 5’ and 3’ unique overhangs of the modular sets, dTALENs with varying number of repeat units (up to 23 bp in this study) can be designed to better meet the criteria in choosing the dual target sites. Our facile modular TALEN assembly method contrasts with another recently reported method that involves several rounds of PCR amplifications and ligations to assemble individual repeats into 12 repeat TAL effectors for gene activation in human cells (16).

A modified plasmid-based yeast SSA lacZ assay was employed to determine activity of the candidate dTALENs before use for final gene targeting. This test system is based on assays developed to initially validate engineered meganucleases and ZFNs (31,32) and was used previously in a preliminary form for TALEN testing (17,18). The original yeast SSA assay relied on inverted repeat DNA targets and engineered homodimeric nucleases (32). For our modified assay, one candidate dTALEN was paired with the proven TALEN AvrXa7-FN (18) to target two adjacent EBEs, one for recognition by the candidate dTALEN and the other for binding of AvrXa7-FN. The paired TALENs derived from the natural TALEs AvrXa7 and PthXo1 were used as a standard. Another advantage of the modified SSA assay is that one EBE and the spacer remain constant so only the second EBE has to be replaced to test a different dTALEN. When testing many dTALENs, this represents a significant (~5×) cost saving due to using much shorter oligonucleotides to create a single (versus double) TALEN site while allowing rapid evaluation of the activity of each individual candidate dTALEN. Moreover, construction of 3’ to 3’ EBE sites containing non-identical DNA sequences avoids the technical challenges associated with cloning and stably maintaining inverted EBE sites containing identical TALE recognition sequences. From a broader perspective, this plasmid-based transient assay should be readily adapted as a facile screening tool in animal and plant cell culture systems.

The 10 dTALENs engineered for this study were designed in pairs to target the native sequences of the yeast genes, *URA3*, *LYS2* and *ADE2*. Each gene has a selectable or easily scored phenotype, so any dTALEN activity should be readily apparent. Expression of dTALEN pairs under appropriate selections indicated that all five dTALEN pairs were functional in creating phenotypic mutant strains whose site-specific DNA alterations were all confirmed by genotyping (Figure 4 and Supplementary Figure S4). The activity of the five dTALEN pairs was comparable to the standard TALEN pair, AvrXa7 and PthXo1. Two pairs of dTALENs also were tested for their ability to mediate HR by targeting *URA3* for gene replacements and compared against HR stimulated by the standard TALEN pair. The results indicated that dTALENs and standard TALENs increase HR at a comparable rate (Figure 4). The low rates of gene disruption *via* NHEJ in present study are probably attributable to the cryptic NHEJ repair pathway in yeast. The NHEJ repair of DSBs in yeast is mostly accurate. For example, repair to DSBs with 4-base overhangs led to an error frequency of only ~1% (43,44). Only cells with mutagenic alterations in the intentionally targeted genes might be recovered (for *URA3* and *LYS2*) or detected (for *ADE2*) by using our procedures. Thus, while the number of dTALENs examined was somewhat limited, these experiments establish that dTALENs are active in vivo in promoting NHEJ- and HR-mediated gene modifications at endogenous loci in an intact, free-living, eukaryotic organism and, thus, verify the power of TALENs for targeted genome editing in eukaryotes beyond the TALEN-mediated gene disruption previously demonstrated in cultured human cells (20).

Taken together, the results of this study indicate that the modular assembly technique is valid, that the yeast SSA assay is a reliable and facile indicator of TALEN activity and that functional dTALENs can target a diverse range of genomic loci. Our study also establishes that yeast, as an intact eukaryotic organism, is a reliable platform to develop and potentially refine engineered nuclease-based technology for targeted genome editing.
The designer TALEN technology described here may overcome a number of critical hurdles (e.g. high cost, limited plasticity and high rates of failure) faced in designing and producing other types of nucleases with DNA targeting capabilities (7,8). The opportunity to build dTALENs with quite long DNA recognition domains bodes well for developing TALENs with exceptionally high accuracy in targeting any gene in any organism, including eukaryotes with highly complex genomes. Such technology has significant potential in experimental biology and medicine and in the development of products with value for human and animal health, agriculture and in a wide range of life sciences industries.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCES
1. Cathomen,T. and Joung,J.K. (2008) Zinc-finger nucleases: The next generation emerges. Mol. Ther., 16, 1200–1207.
2. Carroll,D. (2008) Progress and prospects: Zinc-finger nucleases as gene therapy agents. Gene Ther., 15, 1463–1468.
3. Urnov,F.D., Rebar,E.J., Holmes,M.C., Zhang,H.S. and Gregory,P.D. (2010) Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet., 11, 636–646.
4. Jasin,M. (1996) Genetic manipulation of genomes with rare-cutting endonucleases. Trends Genet., 12, 224–228.
5. Vasquez,K.M., Marburker,K., Intody,Z. and Wilson,J.H. (2001) Manipulating the mammalian genome by homologous recombination. Proc. Natl Acad. Sci. USA, 98, 8403–8410.
6. Bibikova,M., Beumer,K., Trautman,J.K. and Carroll,D. (2003) Enhancing gene targeting with designed zinc finger nucleases. Science, 300, 764.
7. Ramirez,C.L., Foley,J.E., Wright,D.A., Muller-Lerch,F., Rahman,S.H., Cornu,T.I., Winfrey,R.J., Sander,J.D., Fu,F., Townsend,J.A. et al. (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. Nat. Methods, 5, 374–375.
8. Kim,J.S., Lee,H.J. and Carroll,D. (2010) Genome editing with modularly assembled zinc-finger nucleases. Nat. Methods, 7, 91–92.
9. Kim,Y.G., Cha,J. and Chandrasegaran,S. (1996) Hybrid restriction enzymes: Zinc finger fusions to fok I cleavage domain. Proc. Natl Acad. Sci. USA, 93, 1156–1160.
10. Boch,J. and Bonas,U. (2010) Xanthomonas AvrBs3 family-type III effectors: Discovery and function. Annu. Rev. Phytopathol., 48, 419–436.
11. White,F.F., Potnis,N., Jones,J.B. and Koebnik,R. (2009) The type III effectors of xanthomonas. Mol. Plant Pathol., 10, 749–766.
12. Bogdanove,A.J., Schornack,S. and Lahaye,T. (2010) TAL effectors: Finding plant genes for disease and defense. Curr. Opin. Plant Biol., 13, 394–401.
13. Boch,J., Scholze,H., Schornack,S., Landgraf,A., Hahn,S., Kay,S., Lahaye,T., Nickstadt,A. and Bonas,U. (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science, 326, 1509–1512.
14. Moscou,M.J. and Bogdanove,A.J. (2009) A simple cipher governs DNA recognition by TAL effectors. Science, 326, 1501.
15. Morbitzer,R., Romer,P., Boch,J. and Lahaye,T. (2010) Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE) type transcription factors. Proc. Natl Acad. Sci. USA, 107, 21617–21622.
16. Zhang,F., Cong,L., Lodato,S., Kosuri,S., Church,G.M. and Arlotta,P. (2011) Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat. Biotechnol., 29, 149–153.
17. Christian,M., Cermak,T., Doyle,E.L., Schmidt,C., Zhang,F., Hummel,A., Bogdanove,A.J. and Voytas,D.F. (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics, 186, 757–761.
18. Li,T., Huang,S., Jiang,W.Z., Wright,D., Spalding,M.H., Weeks,D.P. and Yang,B. (2010) TAL nucleases (TALENs): Hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. Nucleic Acids Res., 39, 359–372.
19. Mahfouz,M.M., Li,L., Shamimuzzaman,M., Wibowo,A., Fang,X. and Zhu,J.K. (2011) De novo-engineered transcription activator-like effector (TALE) hybrid nucleases with novel DNA binding specificity creates double-strand breaks. Proc. Natl Acad. Sci. USA, 108, 2623–2628.
20. Miller,J.C., Tan,S., Qiao,G., Barlow,K.A., Wang,J., Xia,D.F., Meng,X., Paschon,D.E., Leung,E., Hinkley,S.J. et al. (2011) A TALE nuclease architecture for efficient genome editing. Nat. Biotechnol., 29, 143–148.
21. Kolonin,M.G. and Finley,R.L. Jr (1998) Targeting cyclin-dependent kinases in drosophila with peptide aptamers. Proc. Natl Acad. Sci. USA, 95, 14266–14271.
22. Boeke,J.D., Trueheart,J., Natsoulis,G. and Fink,G.R. (1987) Manipulating the mammalian genome by homologous recombination. Curr. Genet., 30, 14266–14271.
23. Chattoo,B.B., Sherman,F., Azubalis,D.A., Fjellstedt,T.A., Chattoo,B.B., Sherman,F., Azubalis,D.A., Fjellstedt,T.A., Chattoo,B.B., Sherman,F., Azubalis,D.A., Fjellstedt,T.A. et al. (1998) Streptorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol., 154, 164–175.
24. Chattoo,B.B., Sherman,F., Azubalis,D.A., Fjellstedt,T.A., Mehner,D. and Ogor,M. (1979) Selection of lys2 mutants of the yeast Saccharomyces cerevisiae by the utilization of alpha-aminoadipate. Genetics, 93, 51–65.
25. Ugolini,S. and Bruschi,C.V. (1996) The red/white colony color assay in the yeast saccharomyces cerevisiae: Epistatic growth advantage of white ade8-18, ade2 cells over red ade2 cells. Curr. Genet., 30, 485–492.
26. Munberg,D., Muller,R. and Funk,M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Curr. Genet., 28, 150–157.
27. Johnston,M. and Davis,R.W. (1984) Sequences that regulate the expression of yeast genes. Mol. Cell. Biol., 4, 1440–1448.
28. Philippsen,P., Stotz,A. and Scherf,C. (1991) DNA of Saccharomyces cerevisiae. Methods Enzymol., 194, 169–182.
29. Li,H. and Durbin,R. (2009) Fast and accurate short read alignment with burrows-wheeler transform. Bioinformatics, 25, 1754–1760.
30. Li,H., Handsaker,B., Wysoker,A., Fennell,T., Ruan,J., Homer,N., Marth,G., Abecasis,G., Durbin,R. and 1000 Genome Project Data Processing Subgroup. (2009) The sequence Alignment/Map format and SAMtools. Bioinformatics, 25, 2078–2079.
31. Aylon,Y. and Kupiec,M. (2004) DSB repair: The yeast paradigm. DNA Repair, 3, 797–815.
31. Epinat, J.C., Arnould, S., Chames, P., Rochaix, P., Desfontaines, D., Puzin, C., Patin, A., Zanghellini, A., Paques, F. and Lacroix, E. (2003) A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. *Nucleic Acids Res.*, 31, 2952–2962.

32. Townsend, J.A., Wright, D.A., Winfrey, R.J., Fu, F., Maeder, M.L., Joung, J.K. and Voytas, D.F. (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature*, 459, 442–445.

33. Porteus, M.H. and Baltimore, D. (2003) Chimeric nucleases stimulate gene targeting in human cells. *Science*, 300, 763.

34. Alwin, S., Gere, M.B., Guhl, E., Effertz, K., Barbas, C.F. III, Segal, D.J., Weitzman, M.D. and Cathomen, T. (2005) Custom zinc-finger nucleases for use in human cells. *Mol. Ther.*, 12, 610–617.

35. Porteus, M.H. (2006) Mammalian gene targeting with designed zinc-finger nucleases. *Mol. Ther.*, 13, 438–446.

36. Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y.L., Rupniewski, I., Beausejour, C.M., Waite, A.J., Wang, N.S., Kim, K.A. et al. (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.*, 25, 778–785.

37. Szczepak, M., Brondani, Y., Buchel, J., Serrano, L., Segal, D.J. and Cathomen, T. (2007) Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat. Biotechnol.*, 25, 786–793.

38. Cornu, T.I., Thibodeau-Beganny, S., Guhl, E., Alwin, S., Eichtinger, M., Joung, J.K. and Cathomen, T. (2008) DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol. Ther.*, 16, 352–358.

39. Bibikova, M., Golic, M., Golic, K.G. and Carroll, D. (2002) Targeted chromosomal cleavage and mutagenesis in drosophila using zinc-finger nucleases. *Genetics*, 161, 1169–1175.

40. Wright, D.A., Thibodeau-Beganny, S., Sander, J.D., Winfrey, R.J., Hirsh, A.S., Eichtinger, M., Fu, F., Porteus, M.H., Dobbs, D., Voytas, D.F. et al. (2006) Standardized reagents and protocols for engineering zinc-finger nucleases by modular assembly. *Nat. Protoc.*, 1, 1637–1652.

41. Beumer, K., Bhattacharyya, G., Bibikova, M., Trautman, J.K. and Carroll, D. (2006) Efficient gene targeting in drosophila with zinc-finger nucleases. *Genetics*, 172, 2391–2403.

42. Hoover, D.M. and Lubkowski, J. (2002) DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. *Nucleic Acids Res.*, 30, e43.

43. Wilson, T.E. and Lieber, M.R. (1999) Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. *J. Biol. Chem.*, 274, 23599–23609.

44. Moore, J.K. and Haber, J.E. (1996) Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in saccharomyces cerevisiae. *Mol. Cell. Biol.*, 16, 2164–2173.