Chapter 15

Assembly of TALEN and mTALE-Act for Plant Genome Engineering

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Abstract

Transcription activator-like effector (TALE) is a DNA-binding domain that can be paired with a nuclease to create DNA double-strand breaks, or with an effector protein to alter gene transcription. The ability to precisely alter plant genomes and transcriptomes has provided many insights into gene function and has recently been utilized for crop improvement. Easy design and construction of TALE make the tool more accessible to a variety of researchers. Here, we describe two TALE-based systems: transcription activator-like effector nucleases (TALEN), for creating targeted mutations in a gene of interest, and multiplex TALE activation (mTALE-Act), for activating one or a few genes of interest at the transcription level. Assembly of these tools is based on Golden Gate cloning and Gateway recombination, which are cost-effective and streamlined cloning methods.

Key words Transcription activator-like effector, TALEN, mTALE-Act, Genome editing, Golden gate, CRISPR

1 Introduction

Genome engineering utilizes sequence-specific nucleases (SSNs) to create genetic mutations or applies synthetic transcriptional activators or repressors to alter transcription within the genome. It relies on a series of tools for genome editing and transcriptional regulation. The genome editing technology started with meganucleases, zinc-finger nucleases (ZFN), and then transcription activator-like effector nucleases (TALEN), and now CRISPR-Cas systems are the most popular choices due to their ease of use and high efficiency [1, 2]. Harnessing DNA-binding features of ZFN, TALEN, and CRISPR-Cas systems, transcriptional regulation tools can be further developed. In this chapter, we describe methodology to construct two genome engineering tools: TALEN and mTALE-Act (multiplex TALE-Activator).

Transcription activator-like effectors (TALEs) are produced by *Xanthomonas*, a bacterial plant pathogen which excretes TALEs

Pasquale Tripodi (ed.), *Crop Breeding: Genetic Improvement Methods*, Methods in Molecular Biology, vol. 2264, https://doi.org/10.1007/978-1-0716-1201-9_15,

during infection to alter host gene transcription [3]. The discovery that TALEs recognize DNA targets, with one TAL repeat domain for one DNA nucleotide, was an important one in biology [4, 5]. TALEs have a central DNA-binding domain which is composed of many repeats. These repeats are almost identical, except in the 12th and 13th amino acid positions which is referred to as a repeat variable di-residue (RVD). Each RVD binds to a specific nucleotide: HD = C, NG = T, NI = A, and NN = G and A. Decoding the secrets of DNA binding by TALEs immediately shed lights to de novo engineering of DNA-binding domains for any DNA sequences of interest, which led to the development of TALEN [6]. TALEN is based on two TALE-FokI monomers. Directing a pair of such monomers to proximal sequences on both strands of DNA will create a DNA double-stranded break due to FokI dimerization (Fig. 1a). TALEN was successfully used in genome editing in plants such as tobacco (*Nicotiana tabacum*) [7], *Arabidopsis* [8], and rice (*Oryza sativa*) [9]. TALEs can also be fused to a transcriptional activator such as VP64 for engineering synthetic transcriptional activators (Fig. 1b) [10]. Binding upstream of the gene of interest by such synthetic transcriptional activators can result in high gene expression, which is a useful tool for studying gene

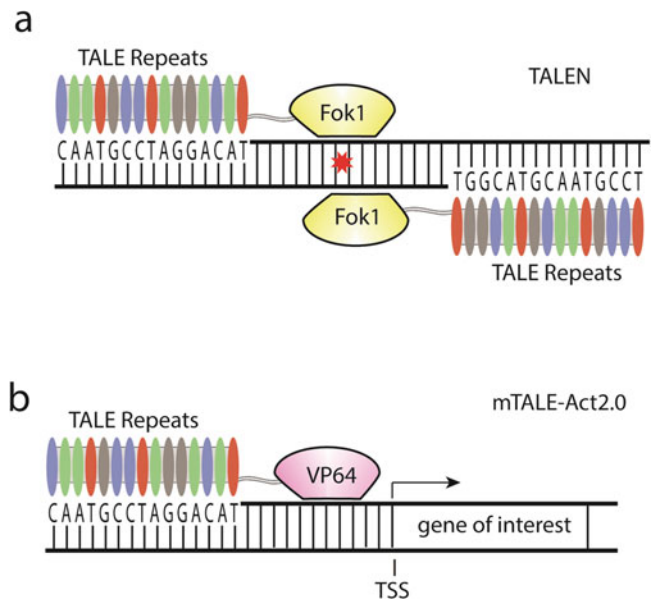

Fig. 1 Diagram of TALEN and mTALE-Act2.0 systems bound to target DNA. Individual RVDs bind to nucleotides and make up the TALE repeats. TALE repeats are fused to an effector protein. (**a**) Fusion of TALE repeats to Fok1 nuclease creates TALEN. Two TALENs are required to create a dimer of Fok1 and induce a DNA double-strand break. (**b**) Fusion of TALE repeats to VP64 activator creates mTALE-Act2.0. Binding upstream of the transcription start site (TSS) activates transcription of the gene of interest

function and regulation. We previously described mTALE-Act system which allows for simultaneous expression of three synthetic TALE-VP64 transcriptional activators for activating up to three genes at once [10].

TALE-based genome engineering is less widespread than CRISPR because it requires more time-consuming construction procedures. However, TALE-based systems are very specific and have unique properties in certain situations. For example, we found that our mTALE-Act system resulted in higher gene expression than CRISPR-Act2.0, which is an improved transcriptional activation system based on CRISPR-Cas9 [10]. Additionally, TALEN is better suited to genome engineering applications that require protein-only nucleases as is the case with mitochondria editing [11]. Here we describe a two-step Golden Gate cloning method for assembling TALE repeats which are sub-cloned into different expression vectors for final assembly of T-DNA vectors based on Gateway recombination for making a TALEN [12] or mTALE-Act system in plants [10].

2 Materials

1. DNA editing computer software such as ApE, Snapgene, and DNA Star and access to TAIR or Genbank.
2. Golden Gate TAL Effector Kit 2.0 from Voytas Lab on Addgene (http://www.addgene.org/kits/voytas-taleffector-goldengatev2/#kit-details). pFUS_A8, pYPQ121, pYPQ127B, and pYPQ202. Plasmids can be found from the Qi Lab on Addgene (http://www.addgene.org/Yiping_Qi/). pZHY013 is also available at Addgene (https://www.addgene.org/36185/).
3. Restriction enzymes *Bsa*I or *Bsa*I-HFv2, *Esp*31/*Bsm*BI, *Eco*RI, *Xba*I, *Bam*HI, *Nhe*I, and *Bgl*II.
4. T4 DNA ligase and 10× T4 DNA ligase buffer.
5. 100 mM Dithiothreitol (DTT).
6. Plasmid-Safe nuclease (Epicentre Biotechnologies, Madison, WI, USA).
7. 25 mM ATP.
8. Taq DNA polymerase, buffer, and dNTPS.
9. Gel electrophoresis equipment.
10. DH5α chemically competent cells.
11. SOC medium: 5 g/L Yeast extract, 20 g/L tryptone, 20 mM dextrose, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride.

12. 50 mg/mL Spectinomycin, carbenicillin/ampicillin, and kanamycin stock.
13. LB plates and liquid media.
14. 40 mg/mL X-gal dissolved in dimethyl sulfoxide or *N*′,*N*-dimethylformamide.
15. 100 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) dissolved in water.
16. Miniprep Kit (QIAprep Spin Miniprep Kit, Qiagen).
17. Primers:
    pCR8_F1: 5′ TTGATGCCTGGCAGTTCCCT 3′
    pCR8_R1: 5′ CGAACCGAACAGGCTTATGT 3′
    TAL_F1: 5′ TGGCGTCGGCAAACAGTGG 3′
    TAL_R2: 5′ GGCGACGAGGTGGTCGTTGG 3′
18. Optional restriction enzymes: *Afl*II, *Bsp*EI.

3 Methods

Two rounds of Golden Gate reactions will be used to assemble 14–16 TALE repeats for recognition of 14–16 bp DNA target sequence. The full 14–16 TALE repeats are broken apart into two segments with each of 7–8 TALE repeats, to be assembled into pFUS_A and pFUS_B vector series, respectively, in the first round of Golden Gate reaction. These two segments will be assembled in the second round of Golden Gate reaction to generate the full-length TALE repeats. Then, fully assembled TALE repeats will be cloned into pYPQ121 and pYPQ127B for multiplex transactional activation by mTALE-Act (Fig. 1b) or cloned into pZHY013 for genome editing by TALEN (Fig. 1a). The final T-DNA vectors are generated by LR Gateway reactions with appropriate attR1-attR2 destination vectors.

3.1 Golden Gate Assembly of TALE Repeats Step 1

1. Download the sequence for *Arabidopsis Cleavage stimulating factor 64 (CSTF64, At1g71800)*, *Glabrous 1 (GL1, At3g27920)*, *RNA-binding protein-defense-related 1 (RBP-DR1, At4g03110)*, and *Alcohol Dehydrogenase 1 (ADH1, At1g77120)* from TAIR (https://www.arabidopsis.org/) or Genbank (https://www.ncbi.nlm.nih.gov/genbank/). The sequence should include ~1000 bp upstream of the transcriptional start site for *CSTF64*, *GL1*, *and RBP-DR1*, which are targeted for transcriptional activation by mTALE-Act. *ADH1* will be targeted for mutagenesis by TALEN. Use a DNA editing software such as ApE, Snapgene, and DNA Star.

2. If designing mTALE-Act for transcriptional activation, select a 14–16 bp target site about ~150 to 350 bp upstream of the gene of interest using TALEN Effector Targeter (https://tale-nt.cac.cornell.edu/node/add/single-tale) of TAL Plasmids Sequence Assembly Tool (http://bao.rice.edu/Research/BioinformaticTools/assembleTALSequences.html). If designing for TALEN for genome editing, select two proximal target sites within an exon, preferably within the first half of the gene to increase the likelihood of creating a knockout. Upload the sequence for the gene of interest and select 14 bp for the minimum and 16 bp for the maximum (*see* **Notes 1** and **2**). We selected the following targets for mTALE-Act: *CSTF64* (5′-ttccttttaaccaaaat-3′), *GL1* (5′-acgtattgatgtgagt-3′), and *RBP-DR1* (5′-ttaatttctcccaact-3′) [10], and the following targets for TALEN: ADH1-left (5′ CCGGATGCTCCTCTT 3′) and ADH1-right (5′ AGTTGTGGTTTGTCT 3′) [8].
3. Select plasmids containing RVD domains for targeting selected sequence. Each RVD corresponds to one nucleotide. The plasmid names correspond to the RVD and the location in the TALE repeat assembly (*see* **Note 3**). Our design uses 8 TALE repeats per vector to increase the efficiency of vector assembly. The first 8 are placed into the pFUS_A8 and then another 6–8 TALE repeats are assembled into pFUS_B(N) using a *Bsa*I-based Golden Gate reaction. The "N" in pFUS_A(N) responds to the number of repeats assembled into the vector. Because pFUS_B houses the final TALE repeat, which is added in a later step, the vector number will be the number of repeats minus 1. For example, one would use pFUS_B7 to house 7 out of 8 TALE repeats, and the last repeat will be added later. The table in **step 4** contains the RVDs used to target our three sequences. The last repeat vector is labeled "LR." pFUS_B8 is used for the repeats of CSTF64, pFUS_B7 for GL1, pFUS_B7 for AtRBP-DR1, pFUS_B7 for ADH1-left, and pFUS_B6 for ADH1-right (*see* **Note 4**).
4. Select the modular plasmids corresponding to the target sequence. The table below describes the vectors used to assemble TALEs for our three target sites.

TALE	RVDs in pFUS_A8 vector							
CSTF64	pNG1	pNG2	pHD3	pHD4	pNG5	pNG6	pNG7	pNG8
GL1	pNI1	pHD2	pNN3	pNG4	pNI5	pNG6	pNG7	pNN8
AtRBP-DR1	pNG1	pNG2	pNI3	pNI4	pNG5	pNG6	pNG7	pHG8
ADH1-left	pHD1	pHD2	pNN3	pNN4	pNI5	pNG6	pNN7	pHD8
ADH1-right	pNI1	pNN2	pNI3	pHD4	pNI5	pNI6	pNI7	pHD8

TALE	RVDs in pFUS_B(N) vector								
CSTF64	pNI1	pNI2	pHD3	pHD4	pNI5	pNI6	pNI7	pNI8	pLR-NG
GL1	pNI1	pNG2	pNN3	pNG4	pNN5	pNI6	pNN7	pLR-NG	
AtRBP-DR1	pNG1	pHD2	pHD3	pHD4	pNI5	pNI6	pHD7	pLR-NG	
ADH1-left	pNG1	pHD2	pHD3	pNG4	pHD5	pNG6	pNG7	pLR-NG	
ADH1-right	pHD1	pNI2	pHD3	pNI4	pNI5	pHD6	pLR-NG		

5. Assemble the modular RVD vectors simultaneously into the pFUS_A8 and pFUS_B(N) vectors using the following Golden Gate reaction (*see* **Notes 5**–**7**):

Golden Gate recipe		Golden Gate program		
Each modular RVD vector pFUS_A8 or pFUS_B(N)	150 ng 75 ng	37 °C 16 °C	5 min 10 min	10×
*Bsa*I	1 μL	50 °C	5 min	
T4 DNA ligase	1 μL	80 °C	5 min	
10× T4 DNA ligase buffer Water	2 μL Up to 20 μL			

6. Cool the reactions on ice and add the following:
   (a) 1 μL 25 mM ATP
   (b) 1 μL Plasmid-Safe nuclease

   Incubate at 37 °C for 1 h (*see* **Note 8**). This step removes all incomplete ligations.

7. Transform 5 μL of reaction into 50 μL of *E. coli* strain DH5α using heat shock or electroporation and rescue with SOC media. Grow at 37 °C for 1 h, and spin and remove supernatant before plating and growing at 37 °C overnight. Use blue/white screening to select correct colonies by using spectinomycin (50 mg/L) LB plates with X-gal and IPTG. If few or no white colonies are observed, then consider replacing the T4 ligase buffer or supplementing with DTT (*see* **Note 6**). Screen both pFUS-A8 and pFUS_B(N) vectors through colony PCR with primers pCR8-F1 and pCR8-R1 using the following recipe and program:

PCR recipe		PCR program		
10× Standard Taq Reaction Buffer	2.5 μL	95 °C	60 s	
10 mM dNTPS	0.5 μL	95 °C	20 s	
pCR8-F1 (10 μM)	0.5 μL	55 °C	30 s	30×

(continued)

PCR recipe		PCR program	
pCR8-R1 (10 μM)	0.5 μL	68 °C	60 s
Taq DNA polymerase	0.1 μL	68 °C	5 min
Water	20.9 μL	10 °C	Hold

The products from the colony PCR will be the full size of the repeat and form a ladder of bands, which is expected from repetitive TALEs (*see* **Note 9**). The full repeat array for 8 RVDs is ~900. The ladder should start around 200 bp and occur every 100 bp. Select colonies and grow overnight in liquid LB with spectinomycin.

8. Purify plasmids from the LB cultures. Correct clones can be confirmed using digestion with *Afl*II and *Xba*I, although this step is optional. RVD sequences can also be confirmed using *Bsp*EI digestion or sequencing with pCR8-F1 and pCR8-R1 primers (*see* **Note 10**).

3.2 Golden Gate Assembly of TALE Repeats Step 2

1. Use a Golden Gate reaction to fuse pFUS_A8, pFUS_B(N), and pLR-NG together into expression vector pZHY500 (*see* **Notes 5**–**7**). This reaction will fuse together the TALE repeats A and B and the last RVD into a backbone that has appropriate restriction enzyme sites for the next cloning step. This reaction will be carried out for each target gene. In our example, the Golden Gate reaction for mTALE-Act targeting *RBP-DR1* will yield pZHY500-1, *CSTF64* vector is pZHY500-2, and *GL1* is pZHY500-3. For TALEN targeting *ADH1*, ADH1-left will yield pZHY500-4 and ADH1-right will yield pZHY500-5.

Golden Gate recipe		Golden Gate program		
pFUS_A8 pFUS_B(N)	150 ng 150 ng	37 °C 16 °C	5 min 10 min	10×
pLR-NG	150 ng	50 °C	5 min	
pZHY500 *Esp*3I/*Bsm*BI T4 DNA ligase 10× T4 DNA ligase buffer Water	75 ng 1 μL 1 μL 2 μL Up to 20 μL	80 °C	5 min	

Plasmid-safe nuclease treatment is not necessary as the expression vector does not have homology with the repeats.

2. Transform into *E. coli* and incubate overnight at 37 °C on LB plates with 50 mg/L carbenicillin or ampicillin. Supplement with X-gal and IPTG for blue and white screening.
3. Select white colonies for inoculation into liquid LB media with carbenicillin/ampicillin. Confirm using colony PCR with primers TAL_F1 and TAL_R2 (*see* **Note 9**). The brightest band

should be the full TALE, which in this example is about 1700 bp. Grow colonies overnight and harvest for miniprep. Further confirmation with digestion and sequencing should be done for the final vectors.

3.3 Assembly of Final T-DNA Vectors for Transcriptional Activation with mTALE-Act

1. Excise the full repeats from pZHY500-1 using restriction enzymes *Xba*I and *Bam*HI and ligate into pYPQ121 using restriction enzyme cloning. This step generates vector pYPQ121-1.
2. Excise the full repeats from pZHY500-2 using restriction enzymes *Nhe*I and *Bgl*II and ligate into pYPQ121-1. This step generates vector pYPQ121-2. Multiplexed mTALE-Acts are separated by a T2A ribosomal skipping motif which allows translation of multiple proteins (the left and right TALE-VP64) from a single transcript [8].
3. Excise the full repeats from pZHY500-3 using restriction enzymes *Xba*I and *Bam*HI and ligate into pYPQ127B (which contains an AtUBQ10 promoter) to generate pYPQ127B-1.
4. The final T-DNA vector is generated through Multisite Gateway recombination with pYPQ121-2, pYPQ127B-1, and destination vector pYPQ202 (*see* **Note 11**) (Fig. 2). This assembles three TALE-VP64 into one vector (*see* **Note 12**). If using one or two TALE-VP64s, use pYPQ140 filler plasmid instead of pYPQ127B.

   Use the following Multisite Gateway recombination recipe (*see* **Note 13**):

Multisite Gateway recombination	
pYPQ121-2	80 ng
pYPQ127B-1	80 ng
pYPQ202	100 ng
LR Clonase II	1 μL
Total volume	7 μL

   Incubate at room temperature overnight.
5. Transform into competent *E. coli* via heat shock or electroporation and plate onto LB media with kanamycin.
6. Select colonies and culture overnight in liquid LB media with kanamycin at 37 °C. Miniprep and verify correct clones by digesting with restriction enzyme *Eco*RI. A correct vector (Fig. 2) will yield bands of 7934, 5423, 3911, 2878, and 350 bp.

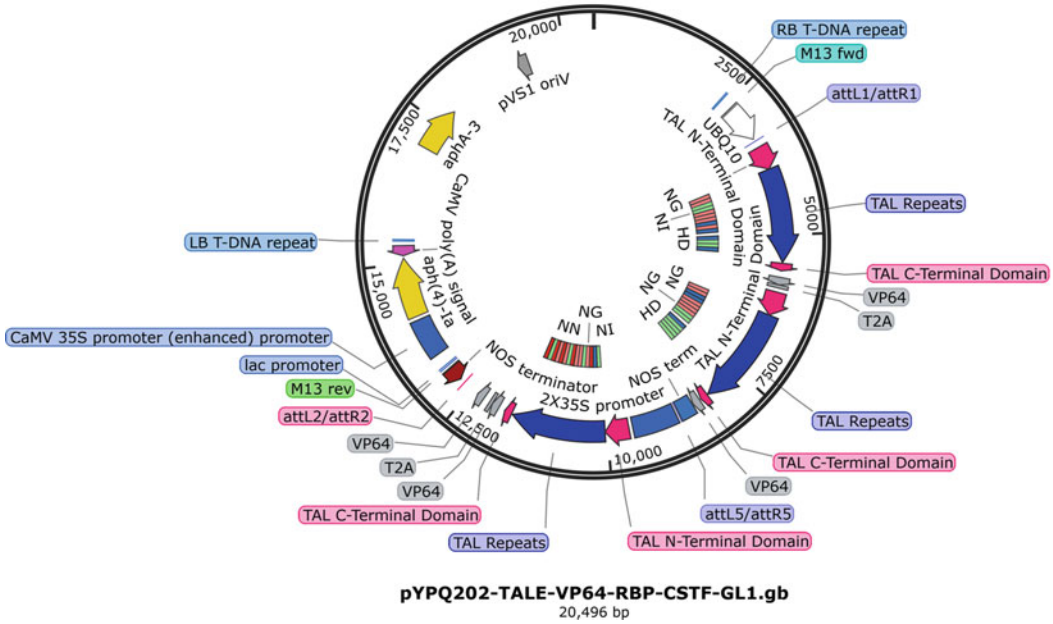

Fig. 2 Vector map of T-DNA vector housing multiplexed mTALE-Act2.0 system targeting *RPB-DR1*, *GL1*, and *CSTF64* for simultaneous transcriptional activation of three genes in *Arabidopsis*

3.4 Assembly of Final T-DNA Vectors for Genome Editing with TALEN

1. Excise the full repeats from pZHY500-4 using restriction enzymes *Xba*I and *Bam*HI and ligate into pZHY013 using restriction enzyme cloning. This step generates vector pZHY013-1.
2. Excise the full repeats from pZHY500-5 using restriction enzymes *Nhe*I and *Bgl*II and ligate into pZHY013-1 at compatible *Xba*I and *Bam*HI sites. This step generates vector pZHY013-2. The T2A ribosomal skipping motif allows translation of the left and right TALEN from a single transcript [8].
3. The final T-DNA vector is generated through Multisite Gateway recombination with pZHY013-2 and pYPQ202 (*see* **Note 11**) (Fig. 3). This assembles two TALEN into one vector driven by an AtUBQ10 promoter (*see* **Note 12**).

   Use the following Multisite Gateway recombination recipe (*see* **Note 13**):

Multisite Gateway recombination	
pZHY013-2	80 ng
pYPQ202	100 ng
LR Clonase II	1 μL
Total volume	7 μL

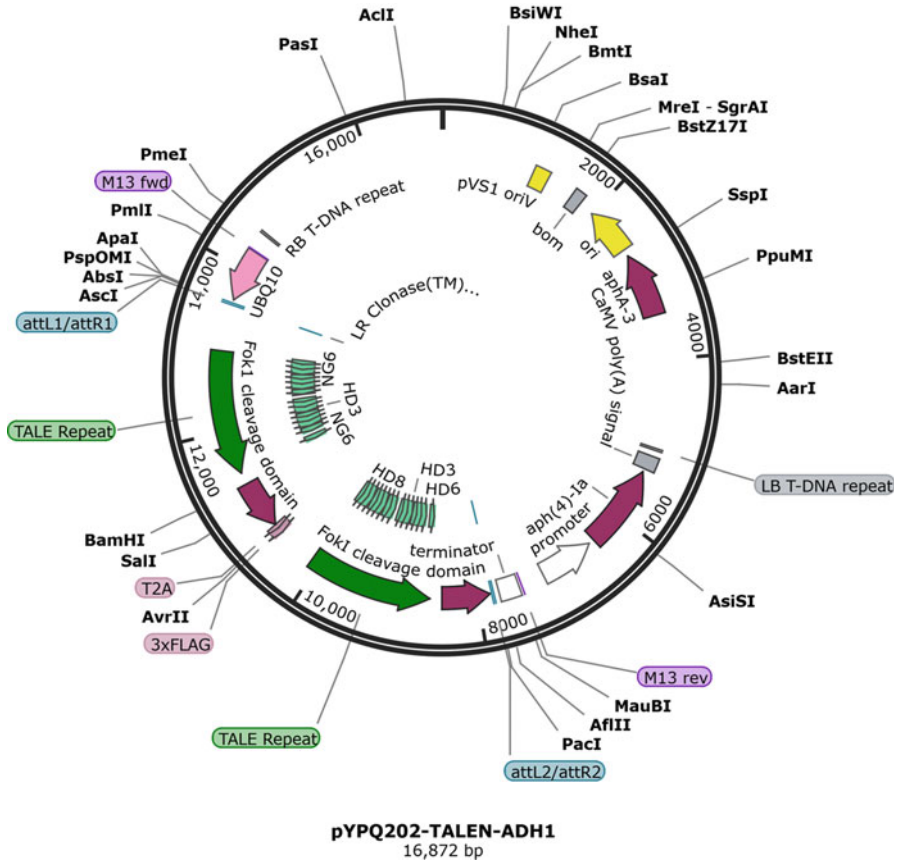

Fig. 3 Vector map of T-DNA housing two TALENs targeting *ADH1* in *Arabidopsis*

Incubate at room temperature overnight.

4. Transform into competent *E. coli* via heat shock or electroporation and plate onto LB media with kanamycin.
5. Select colonies and culture overnight in liquid LB media with kanamycin at 37 °C. Miniprep and verify correct clones by digesting with restriction enzyme *Eco*RI. A correct vector (Fig. 3) will yield bands of 7934, 5486, 2878, 485, and 350 bp.

4 Notes

1. We recommend using T as the first base of a TALE target site or at the −1 position [13]. This is sometimes referred to as the "upstream base." We also recommend using T as the last base of a TALE target site.
2. Streubel et al. showed that NH binds the nucleotide G more specifically than NN, although TALEN Effector Targeter has

the option to select either RVD for the design. We recommend using NN to bind to G since higher binding affinity has been observed with NN than NH [10, 14].

3. *E. coli* housing vector pHD2 is slow growing, and it is unclear why. Additionally, plasmid yields from *E. coli* strain DH5α are higher than from strain DH10B. Plasmids from Addgene are in one of these two strains. It is unclear what mechanism is behind this difference.
4. The vectors in pFUS_B are numbered starting with 1, even though they would follow the RVDs in the pFUS_A(N) vector. In this example, this would be the ninth position in the TALE assembly.
5. Use *Bsa*I which works better in the Golden Gate reaction than *Bsa*I-HF New England Biolabs has released, *Bsa*I-HFv2 (NEB #R3733), which is designed to work well in Golden Gate reactions. This enzyme was not used in this study and we cannot comment directly on the efficiency of the enzyme.
6. Use fresh T4 DNA ligase buffer as it can lose potency and is often the cause of an inefficient Golden Gate reaction. We recommend aliquoting ligase buffer into ~25 μL volumes. A simple way to determine if T4 DNA ligase buffer has gone bad is the absence of the sulfuric smell of dithiothreitol (DTT) in the buffer. If there is no smell, DTT can be added to 1 mM concentration in the final reaction.
7. Golden Gate reactions can be performed in 10 μL reactions to save reagents, but difficult reactions may require 20 μL reactions.
8. The plasmid-safe protocol recommends inactivating the enzyme by heating the reaction to 70 °C for 30 min, but this is an optional step.
9. Select between 3 and 10 colonies to screen with colony PCR. When screening via colony PCR, correct colonies can appear as a smear on the agarose gel. However, there should be a slightly brighter band at the lengths of the repeats within the smear.
10. Sequencing TALEs can be difficult due to the repeats but it is important to confirm correct sequences. Rarely, mutations can occur that affect activity.
11. Multisite Gateway recombination is sensitive to concentration; attention should be paid to the concentration of the vectors, which should be diluted if necessary.
12. It is easy to use a different promoter to drive the expression of mTALE-Act or TALEN. Instead of using pYPQ202, other destination vectors containing different promoters with attR1-attR2 Gateway recombination sites may be used.

13. We have found that Invitrogen Gateway LR Clonase II enzyme mix works well and it is not necessary to use Multisite-Gateway Pro kit which is more expensive.

Acknowledgments

This work is supported by NSF Plant Genome ECA-PGR Award (IOS-1758745) to the University of Maryland, College Park.

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